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April 3, 2003
Date

Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Roth

Serial No.: 09/447,681

Filed: November 23, 1999

For: ADENOVIRUS p53 COMPOSITIONS AND METHODS

Group Art Unit: 1632

Examiner: Crouch, Deborah

Atty. Dkt. No.: INRP:003--2

BRIEF ON APPEAL

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Roth	Group Art Unit: 1632
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For: ADENOVIRUS p53 COMPOSITIONS AND METHODS	

APPEAL BRIEF

BOX AF
Commissioner of Patents
Washington, D.C. 20231

Commissioner:

Appellant hereby submits an original and three copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated September 27, 2002. The Notice of Appeal was received by the Patent and Trademark Office on January 3, 2003, as evidenced by the stamped postcard. A request for a one-month extension of time is included herewith, along the required fee for the extension of time and the fee for filing the appeal brief.

Should any other fees be due, or the attached fee be deficient or absent, the Commissioner is authorized to withdraw the appropriate fee from Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/INRP:003--2. Please date stamp and return the enclosed postcard to evidence receipt of this document.

I. REAL PARTY IN INTEREST

The real parties in interest are the assignee, Board of Regents, University of Texas System, Austin, TX, and the licensee, Introgen Therapeutics, Inc., Austin, Texas.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-30 were filed with the original application, Serial No. 07/960,513, on October 13, 1992, and claims 31-65 were subsequently added by amendment in the parent case. The original application is a CIP of 07/665,538, filed on March 6, 1991. This application is a continuation of the original application.

In a Preliminary Amendment filed with this application, claims 1-65 were canceled and claims 66-85 were added. In a Response under 37 C.F.R. § 1.116 to the Office Action dated August 14, 2000, claims 66, 68-85 were cancelled and claim 67 was amended. In a Preliminary Amendment filed with a Continued Prosecution Application on May 13, 2002, claims 86-89 were added. Thus, claims 67 and 86-89 are pending and appealed. A copy of the appealed claims is attached as Exhibit 1 to this brief.

IV. STATUS OF AMENDMENTS

Appellant filed a preliminary amendment on May 13, 2002, with a Continued Prosecution Application, which the Office Action indicates has been entered, as is show in the pending claims attached as Exhibit 1. No additional amendments have been made herewith.

V. SUMMARY OF THE INVENTION

The present invention is drawn to an adenovirus vector with a wild type p53 gene under the control of a CMV promoter. Specification at page 15, lines 3-5.[1] Other aspects of the invention are directed to an adenovirus vector with a wild type p53 gene under the control of a promoter. The promoter may be a β-actin promoter, a SV40 promoter, or a RSV promoter. Specification at page 14, lines 27-35 through page 15, lines 1-5.

VI. ISSUES ON APPEAL

- Is claim 67 properly rejected over claims 22, 29, and 32-34 of Application No. 09/668,532 as unpatentable under the judicially created doctrine of obviousness-type double patenting?
- Are claims 67 and 86-89 properly rejected over claims 1-3, 5, 8-10, 12, and 15-18 of U.S. Patent No. 6,410,010 as unpatentable under the judicially created doctrine of obviousness-type double patenting?
- Are claims 67, and 86-89 properly rejected for lack of written description under 35 U.S.C. §112, first paragraph, and thus properly denied the benefit of its October 23, 1992 priority date?
- Are claims 67, and 86 properly rejected under 35 U.S.C. §102(b)?
- Are claims 86-89 properly rejected for obviousness under 35 U.S.C. §103(a)?

VII. GROUPING OF THE CLAIMS

The claims do not stand or fall together. Claims 67, 87, 88, and 89 each recite a different promoter, while claim 86 recites a promoter but does not recite a specific promoter. All of the

claims have been rejected as lacking an adequate written description and as obvious. Claim 86 stands separately from claims 67, 87, 88, and 89 because it does not recite a specific promoter, and thus, the written description requirement for it is different than the claims that do recite a specific promoter. Furthermore, claim 86, unlike claims 87, 88, and 89, has been rejected as anticipated over the reference of Liu *et al.* Therefore, claim 86 stands or falls separately from claims 67, 87, 88, and 89.

Claims 67, 87, 88, and 89 each have a different set of references cited again them in rejecting them as obvious. The arguments with respect to each set is different and thus, the issue of patentability is different. Therefore, each of claims 67, 87, 88, and 89 stands or falls separately from the other claims.

VIII. SUMMARY OF THE ARGUMENT

The claims should not be rejected under the obviousness-type double patenting rejection because the cited patent and patent application have a priority date after the claimed priority date of the present application, and thus, the submission of terminal disclaimers is not appropriate.

It is the Examiner's position that the claims are not fully described and that the application is not entitled to its priority date. Of particular concern is a adenovirus vector comprising a wild type p53 gene under the control of a promoter or of a specific promoter. However, Appellant was in possession of the adenovirus vector comprising a wild type p53 gene under the control of a promoter at the time of the invention. Portions of the specification make this clear, as does the submitted declaration of Lou Zumstein, Ph.D., a person of ordinary skill in the art. Thus, the claims are supported by an adequate written description and they are entitled to the benefit of this application's priority date.

Some of the claims have been rejected as anticipated based on the Liu *et al.* reference. This reference, however, is not prior art against the claims because the claims are entitled to the benefit of their priority date.

All of the claims have been rejected as obvious in view of different sets of references. The rejections are improper because there is no motivation or suggestion to combine references to teach all of the elements of the claimed invention. As such, the claims are not obvious.

IX. ARGUMENT

A. "Substantial Evidence" Required

As an initial matter, Appellants note that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 165 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Provisional Obviousness-Type Double Patenting Rejection over 09/668,532

The Action provisionally rejects claim 67 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 22, 29, and 32-34 of co-pending Application No. 09/668,532 ('532 application) (INRP:005USC2). A terminal disclaimer is submitted herewith (Exhibit 2); accordingly, this rejection should be withdrawn.

C. Obviousness-Type Patent Rejection ver U.S. Patent No. 6,410,010

The Action provisionally rejects claims 67 and 86-89 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-3, 5, 8-10, 12, and 15-18 of issued U.S. Patent No. 6,410,010. A terminal disclaimer over this patent is included herewith (Exhibit 3); accordingly this rejection should be withdrawn.

D. Claims 67, and 86-89 Are Properly Described

The Action rejects claims 67 and 86-89 as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. The Action argues that the claims contain "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." Action at page 5. The Action also contends that the instant specification does not contemplate adenoviral vectors comprising a wild type p53 gene operably linked to a promoter or to a specific promoter. The Action admits there is "generic disclosure to other promotor/vector constructs" but contends that there is no specific disclosure of another vector construct. The Action then separately considers each passage from the Specification cited by the Appellant and concludes the passages fails "to provide written description for the claimed invention." It further alleges that at no place in the specification is the invention clearly set forth so that the reader would realize what the Appellant perceived as his invention at the time of filing. Appellant respectfully traverses this rejection.

The written description requirement is whether the "description clearly allows persons of ordinary skill in the art to recognize that he or she invented what is claimed." MPEP 2163.02 (citing *In re Gosteli*, 872 F.2d 1008, 1012, 10 U.S.P.Q.2d 1614, 1618 (Fed. Cir. 1989)). Appellant contends that it is clear that the specification describes what is claimed in rejected claims 67, 86-89. The claims are generally directed to an "adenovirus vector comprising a wild type p53 gene under the control of a promoter." The written description of this application supports this claim and claims 67 and 87-89, which recite specific promoters. The specification makes clear that the inventor was in possession of the claimed invention:

- "In one specific embodiment, the invention concerns vector constructs for introducing wild type p53 genes (wt-*p53*) into affected target cells suspected of having mutant *p53* genes. These embodiments involve the preparation of a gene expression unit wherein the wt-*p53* gene is placed under the control of the β-actin promoter, and the unit is positioned in a reverse orientation into a retroviral vector." Specification at page 9, lines 6-12.

- In Example III, "The *p53* cDNA with its β-actin promoter was cloned into the LNSX retroviral vectors in ***both*** orientations." Specification at page 61, lines 29-30 (emphasis added).

- "While this affect [sic] was observed using the β-actin promoter and a retroviral expression vector, the inventors believe that this phenomenon ***will be applicable to other promoter/vector constructs for application in gene therapy***." Specification at page 8, line 25 to page 9, line 4 (emphasis added).

- "In addition to retroviruses, it is contemplated that ***other vectors can be employed, including adenovirus***...." Specification at page 14, lines 21-23 (emphasis added).

- "While the **β-actin promoter** is preferred, the invention is by no means limited to this promoter and one may also mention by way of example promoters derived from **RSV**, N2A, LN, LNSX, LNSN, **SV40**, LNCX or **CMV**." Specification at page 15, lines 1-4 (citations omitted) (emphasis added).

- "***Generally speaking***, such a promoter might include either a human cellular or viral promoter. While the β-actin promoter is preferred the invention is by no means limited to this promoter...." Specification at page 14, line 35-page 15, lines 2 (emphasis added).

- "While the retroviral construct aspect of the invention concerns the use of a β-actin promoter in reverse orientation, there is no limitation on the nature of the selected gene which one desires to have expressed. Thus, the invention concerns the use of antisense-encoding constructs ***as well as 'sense' constructs that encode a desired protein.***" Specification at page 16, lines 5-10.

Therefore, the Specification makes clear that 1) p53 sense constructs are contemplated in both orientations; 2) any discussion about antisense constructs applies to "sense" constructs such as p53; 3) constructs can be retroviral, but they may also be adenovirus constructs, and thus, are not limited to retroviruses; 4) promoters are discussed both generally and in the context of antisense constructs, in addition to CMV, RSV, and SV40 being specifically mentioned; and finally, 5) because an adenovirus can be used instead of retrovirus and since constructs are not limited to antisense constructs, applying equally to sense constructs, there is adequate written description for an "adenovirus vector comprising a wild type p53 gene under the control of a promoter," as well as for vectors with a CMV promoter.

In addition to the Declaration of Dr. Lou Zumstein (Exhibit 4), submitted with the Response filed on October 18, 2001, Appellant submitted the Declaration of Dr. Philip Hinds (Exhibit 5) with the CPA filed on May 13, 2002. Both of these constitute evidence from a person of ordinary skill in the art to support the contention that the Appellant was in possession of the claimed invention at the time the priority application was filed. Appellant contends that the Action has not rebutted the evidence submitted by persons of ordinary skill in the art to maintain the rejection of these claims. Evidence, as opposed to examiner argument, should be required to meet the "preponderance of the evidence" standard set forth in MPEP § 2163.04. The declarations and the identified portions of the specification show the written description requirement has been met. Accordingly, Appellant respectfully requests this rejection be withdrawn.

The Action contends that in the places where adenovirus or promoters claimed are disclosed, "each such disclosure is within the context of antisense RNA production." Office Action page 6. Appellant denies that adenoviruses are discussed in the application only in the context of antisense embodiments. The paragraph in which the Specification discloses that other vectors such as adenovirus can be used instead of a retrovirus begins, "In broader aspects of the invention, a preferred approach will involve the preparation of retroviral vectors which incorporate nucleic acid sequences encoding the desired construct, once introduced into the cell to be treated...." Specification at page 14, lines 9-12. The use of adenovirus is discusses in the context of "broader aspects of the invention," and retroviruses and antisense constructs are but examples of aspects of the invention. Similarly, as quoted above, the following paragraph discussing promoters indeed recites particular embodiments of the invention, such as antisense; however, it says, "***Generally speaking***, such a promoter might include either a human cellular or viral promoter. While the β-actin promoter is preferred the invention is by no means limited to this promoter...." Specification at page 14, line 35-page 15, lines 2 (emphasis added).

Because the Specification indicates to a skilled artisan that the inventor was in possession of the claimed invention at the time the application was filed, Appellant respectfully requests this rejection be withdrawn. Furthermore, because the application complies with 35 U.S.C. §112, the claims are entitled to the benefit of their priority date of October 13, 1992. 35 U.S.C. 120.

E. Claims 67 and 86 Are Not Anticipated under § 102 (b)

The Action rejects claims 67 and 86 as unpatentable over Liu *et al.* (1994) ("Liu") (Exhibit 6) based on 35 U.S.C. § 102(b). It contends that Liu anticipates the claimed invention. Appellant respectfully traverse this rejection.

The present application claims priority to 07/960,513 ('513 application), filed October 13, 1992. Moreover, the Action levies a written description rejection based on the '513 application. Appellant once again asserts a claim of priority for the present application to the '513 application. As is discussed above, Appellant is entitled to a priority date of October 13, 1992.

Accordingly, claims 67 and 86 are not anticipated by Liu because it is not prior art against the claimed invention. Liu was published in 1994, while the present application is entitled to a priority date that precedes the Liu publication date. Because Liu is not prior art against the application, it cannot anticipate the claimed invention. Consequently, Appellant respectfully requests this rejection be withdrawn.

F. Claims 86-89 Are Nonobvious under 35 U.S.C. § 103 (a)

The Action rejected claims 86-89 under 35 U.S.C. § 103 (a) as being unpatentable over Chen *et al.* (Chen) (Exhibit 7) in view of Wilkinson *et al.* (Wilkinson) (Exhibit 8), Colicos *et al.* (Colicos) (Exhibit 9), Rajan *et al.* (Rajan) (Exhibit 10), and Hitt *et al.* (Hitt) (Exhibit 11). It alleges that the Chen references teaches that wild-type p53 is expressed in transduced cells and that these cells fail to form tumors in nude mice. The Action admits that this reference does not teach an adenoviral vector comprising a wild-type p53 gene under the control of a promoter, but instead, relies upon Wilkinson as allegedly teaching an adenovirus expression system utilizing a CMV promoter to regulate expression of *lacZ*. The Action also contends that Colicos teaches an adenovirus vector containing the RSV promoter, that Rajan teaches an adenoviral vector containing an SV40 promoter, and that Hitt teaches an adenovirus vector with a human actin promoter. The Action further argues that the motivation to make the claimed vectors is provided by the Chen reference's statement that expression of p53 in cells lacking functional p53 reverts

the cells' transformed phenotype and suggests possible clinical use of p53 gene replacement. Appellant respectfully traverses this rejection.

The Federal Circuit held in *In re Mills*, 916 F.2d 680, 682 (Fed. Cir. 1990), that the mere fact that combination or modification of a reference or references is possible does not establish obviousness of the resultant combination unless the prior art also suggests the desirability of the combination, *i.e.*, unless the prior art provides motivation to produce the resultant combination. *Id.*; *see also* MPEP § 2143.01, page 2100-91. Alternatively, Federal Circuit caselaw requires motivation ***to combine references.*** "To combine references (A) and (B) properly to reach the conclusion that the subject matter of a patent would have been obvious, case law requires that there must be some teaching, suggestion, or inference in either reference (A) or (B), or both, or knowledge generally available to one of ordinary skill in the relevant art that would lead one skilled in the art to combine the relevant teachings of references (A) and (B)." *Ashland Oil, Inc. v. Delta Resins & Refractories, Inc.*, 776 F.2d 281, 227 U.S.P.Q. 657 (Fed. Cir. 1985). The Action has not satisfied either. It provides neither the basis for combining the Chen reference with the Wilkinson reference or the basis for combining the adenovirus vector of Wilkinson with the wt p53 gene of Chen. The Chen reference purportedly discloses a retroviral vector comprising a wild-type p53 gene sequence. The Action states, "Motivation is provided by Chen et al stating that expression of p53 in cells [sic] Saos cells which lack functional p53 reverts the transformed phenotype, and that such suggests possible clinical use of p53 gene replacement." This basis is simply not sufficient for a *prima facie* case of obviousness. This is particularly true given that there is no cited evidence that a person of ordinary skill in the art would have considered using adenovirus in a therapeutic context. As discussed in the Chen reference (*e.g.*, page 1579), retroviruses integrate into the genome, unlike adenoviruses. Thus, Chen mentions

replacing mutated tumor suppressors with wild-type versions, which is possible in the context of retroviruses, but not adenoviruses. Chen reference at page 1579 ("These shared properties of RB and p53 reinforce the tumor suppressor gene concept, including the possible clinical use of their ***replacement*** in appropriate tumor cells.") (Emphasis added.) There is no suggestion in any of the cited references that one should substitute the retroviral vector of Chen with the adenoviral vector of Wilkinson for the *p53* construct, any more than there is the suggestion to make the retroviral vector of Chen with the ***lacZ*** **gene** of Wilkinson. Accordingly, there is no evidence that a person of ordinary skill in the art would have combined the expression of p53 from the Chen reference with the use of an adenovirus from the Wilkinson reference.

Furthermore, "[i]t is impermissible within the framework of 35 U.S.C. § 103 to pick and choose from any on reference only so much of it as will support a given position to the exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one skilled in the art." *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc.*, 230 U.S.P.Q. 416 (Fed. Cir. 1986).

Once again, Chen allegedly teaches a retrovirus vector with a wild-type *p53* gene under the control of an LTR promoter; Wilkinson allegedly teaches an adenovirus vector with a *lacZ* gene under the control of a CMV promoter. The references of Colicos, Rajan, and Hitt are cited respectively for the use of an adenoviral vector comprising an RSV promoter, the use of an adenovirus with an SV40 promoter, and the use of an adenovirus with a β-actin promoter. In each case the promoter drives the expression of a gene that is ***not*** a p53 gene and that is different from the other cited references. The Action merely points to references that disclose elements of the claimed invention and asserts it would have been obvious to the ordinary artisan at the time of the invention to use an adenoviral vector comprising a human wild-type *p53* gene operably

linked to a specific promoter. Such an artisan would have to pick out *p53* as the gene to be expressed in an adenovirus vector and under the control of specific promoters. Appellant contends that the absence of a suggestion or motivation to combine renders any *prima facie* case of obviousness based on these references to be fatally flawed. There is nothing in Chen that suggests or motivates its combination with any of Wilkinson, Colicos, Rajan, or Hitt to produce the claimed invention. Furthermore, the references of Wilkinson, Colicos, Rajan, and Hitt do not suggest that any of them be combined with Chen.

If anything, the references of Wilkinson, Colicos, Rajan, and Hitt teach away from the claimed invention because they fail to mention p53, and they discuss expression of other genes, none of which is a therapeutic gene. In fact, several of the genes were believe to contribute to transformation of cells. As the Action acknowledges, Wilkinson teaches the expression of ***lacZ***, not p53, under the control of a promoter. Similarly, Colicos teaches expressing the ***denV*** gene product, a DNA glycosylase from bacteriophage T4. Rajan discusses expression of **SV40 small-t antigen**, which is a viral protein from the SV40 virus. Finally, Hitt reports the expression of **E1A**, an adenovirus oncoprotein. At the time the application was filed, both E1A and small-t antigen were known to play roles in cell transformation—a process that is the opposite of tumor suppression. *See* Hitt at page 667 and Rajan at page 6557.

Patent law states, "The relevant portions of a reference include not only those teachings which would suggest particular aspects of an invention to one having ordinary skill in the art, but also those teachings which would lead such a person away from the claimed invention." *In re Mercier,* 185 U.S.P.Q. 774, 778 (C.C.P.A. 1975). The relevant portions of the references teach away from using adenoviruses to express p53. Thus, there is no suggestion to combine the teachings of Chen with any of the references of Wilkinson, Colicos, Rajan, and Hitt.

Moreover, because there is no mention of p53 in the context of these references, and thus, no motivation or suggestion to combine them with Chen to produce the claimed invention, this combination of references does not render obvious the claimed invention. A proper *prima facie* case of obviousness has not been made. Therefore, this rejection should be withdrawn.

Accordingly, Appellant contends that the vector claims are patentable over the cited references, and respectfully requests the withdrawal of this rejection for claims 86-89.

X. CONCLUSION

It is respectfully submitted, in light of the above, that all pending claims are fully described and thus satisfy 35 U.S.C. §112, first paragraph; all pending claims are entitled to an earlier priority date; and all pending claims are non-obvious over the cited art and thus satisfy 35 U.S.C. §§ 102 and 103 (a). Therefore, Appellant requests that the Board reverse the pending rejections and allow the claims.

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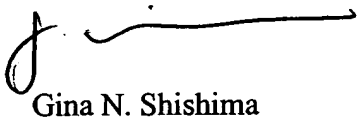
Respectfully submitted,



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Date: April 3, 2003

EXHIBIT 1: PENDING CLAIMS

67. (Amended) An adenovirus vector comprising a wild type p53 gene under the control of a CMV promoter.

86. An adenovirus vector comprising a wild type p53 gene under the control of a promoter.

87. The vector of claim 86, wherein the promoter is the β-actin promoter.

88. The vector of claim 86, wherein the promoter is the SV40 promoter.

89. The vector of claim 86, wherein the promoter is the RSV promoter.

PTO/SB/25 (10-00)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING SECOND APPLICATION	Docket Number (Optional) INRP:003–2

In re Application of: Roth

Application No.: 09/447,681

Filed: November 23, 1999

For: ADENOVIRUS P53 COMPOSITIONS AND METHODS

The owner*, Board of Regents, The University of Texas System, of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application, which would extend beyond the expiration date of the full statutory term defined in 35 U.S.C. 154 to 156 and 173 as shortened by any terminal disclaimer filed prior to the grant of any patent granted on pending second Application Number 09/668,532, filed on 9/21/2000, of any patent on the pending second application.The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the second application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 to 156 and 173 of any patent granted on the second application, as shortened by any terminal disclaimer filed prior to the patent grant, in the event that any such granted patent: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

Check either box 1 or 2 below, if appropriate.

1. ☐ For submissions on behalf of an organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ☑ The undersigned is an attorney or agent of record.

Signature 4/3/03 Date

Gina N. Shishima, Reg. No. 45,104
Typed or printed name

☑ Terminal disclaimer fee under 37 CFR 1.20(d) is included.

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*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner). Form PTO/SB/96 may be used for making this statement. See MPEP § 324.

PTO/SB/26 (10-00)
Approved for use 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A PRIOR PATENT	Docket Number (Optional) INRP:003–2

In re Application of: Roth

Application No.: 09/447,681

Filed: November 23, 1999

For: ADENOVIRUS P53 COMPOSITIONS AND METHODS

The owner*, Board of Regents, The University of Texas System, of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application, which would extend beyond the expiration date of the full statutory term defined in 35 U.S.C. 154 to 156 and 173, as presently shortened by any terminal disclaimer, of prior Patent No. 6,410,010. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 to 156 and 173 of the prior patent, as presently shortened by any terminal disclaimer, in the event that it later: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

Check either box 1 or 2 below, if appropriate.

1. ☐ For submissions on behalf of an organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ☑ The undersigned is an attorney or agent of record.

Signature 4/3/03 Date

Gina N. Shishima, Reg. No. 45,104
Typed or printed name

☑ Terminal disclaimer fee under 37 CFR 1.20(d) included.

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October 18, 2001
Date

Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Jack A. Roth

Serial No.: 09/447,681

Filed: November 23, 1999

For: ADENOVIRUS p53 COMPOSITIONS AND METHODS

Group Art Unit: 1632

Examiner: Crouch, D.

Atty. Dkt. No.: INRP:003--2

SUBSTITUTE DECLARATION OF LOUIS ZUMSTEIN, PH.D

UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Louis Zumstein, Ph.D., declare the following:

1. I am the Director of Research at Introgen Therapeutics in Houston, Texas. I have a Ph.D in Biochemistry and Molecular Biology from Harvard University. I have done extensive research on tumor suppressor genes, including p53, and their delivery to cells via viral vectors. I have authored five scientific papers on these topics. My *curriculum vitae* is attached as Exhibit 1.

2. I am familiar with the level of skill of scientists working in the field of gene therapy as of the October, 1992 priority date of the referenced application.

3. I have read the specification and pending claims 66-70 (as amended) for the above-referenced case. Claim 66 reads, "An adenovirus vector comprising a wild type p53 gene under the control of a promoter." Claim 67 reads, "The vector of claim 66, wherein the promoter is a CMV promoter. Claims 68-70 cover other promoters, specifically β-actin, SV40, and RSV. Claim 71 reads, "The vector of claim 66, wherein the wild type p53 gene is a human gene." A copy of these claims are attached as Exhibit 2.

4. From reading the specification, it is clear to me that had a person skilled in molecular biology and tumor suppressor genes read this specification in October of 1992, it would readily described to such a person an adenoviral vector encoding the p53 gene under a promoter, including a CMV, β-actin, SV40, and RSV promoter. This understanding is supported by the specifications on page 6, lines 33-35, which states "Another important 'oncogene' is the gene encoding p53....one of the most common targets for genetic abnormalities in human tumors"; on page 14 lines 22-23, which states "other vectors ... including adenovirus ...."; and page 15, lines 1-4, which states, "While the β-actin promoter is preferred the invention is by no means limited to this promoter, and one may also mention by way of example promoters derived from RSV...SV40...or CMV."

5. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable

by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

August 20/2001
Date

Louis Zumstein, Ph.D.

CERTIFICATE OF EXPRESS MAILING	
NUMBER	EL 794536973 US
DATE OF DEPOSIT	May 13, 2002

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Jack A. Roth

Serial No.: 09/447,681

Filed: November 23, 1999

For: ADENOVIRUS p53 COMPOSITIONS AND METHODS

Group Art Unit: 1632

Examiner: Crouch, D.

Atty. Dkt. No.: INRP:003--2

DECLARATION OF PHILIP W. HINDS, PH.D UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Philip W. Hinds, Ph.D., declare the following:

1. I am an Associate Professor in the Pathology Department at Harvard Medical School. I have a Ph.D in Molecular Biology from Princeton University. My doctoral dissertation focused on p53 and its role as an anti-oncogene. At Harvard, I have done research on tumor suppressors, including p53 and Rb. I have authored a number of scientific papers on these topics. My *curriculum vitae* is attached as Exhibit 1. Based on my knowledge of the scientific literature and my own research, I am familiar with the

level of skill of a person with ordinary skill in the p53 field in the 1992 to 1993 time frame.

2. I have reviewed the specification of the instant application.

3. It is my opinion that the specification indicates that the inventors contemplated that adenovirus could be substituted for retrovirus as a vector for wild-type p53 under the control of a promoter. I base this conclusion on the wording of the specification, which makes it clear that the inventors envisioned and planned for an adenovirus vector encoding wild-type p53 under the control of a promoter. In particular, I base my conclusions on page 9, lines 6-8 and page 14 lines 21-23 of the specification.

4. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

3 May 2002
Date

Philip W. Hinds, Ph.D.

Principal Investigator/Program Director (Last, first, middle): Hinds, Philip W.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. DO NOT EXCEED FOUR PAGES.

NAME	POSITION TITLE
Philip W. Hinds, Ph.D.	Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Maine, Orono, Maine	BS	1983	Biochemistry
University of Maine, Orono, Maine	MS	1984	Biochemistry
Princeton University, Princeton, New Jersey	Ph.D.	1989	Molecular Biology
Whitehead Institute, Cambridge, MA	postdoc	1993	Molecular Biology

Pr fessional Experience

1993 Instructor of Biochemistry, University of Maine, Department of Biochemistry

1985-1989 Ph.D. Molecular Biology (October 1989), Princeton University, Princeton, NJ 08544. Thesis advisor: Arnold J. Levine, Ph.D.; Project: Function of the p53 oncogene/tumor suppressor

1989-1993 Postdoctoral Fellow, Whitehead Institute, Cambridge, Massachusetts
Laboratory advisor: Robert A. Weinberg, Ph. D.

1993-2000 Assistant Professor, Department of Pathology, Harvard Medical School, Boston, MA

1997-2000 American Cancer Society Cell Cycle Study Section, Chair 1998-2000

2000- Associate Professor, Department of Pathology, Harvard Medical School, Boston, MA

Honors and Awards

1983 Frederick H. Radke Scholar in Biochemistry (Department of Biochemistry, University of Maine)
1984 George F. Dow Award (College of Life Sciences and Agriculture, University of Maine)
1985 Baxter-Travenol Fellowship
1986 U.S. Public Health Service National Research Service Award CA09528
1988-1989 New Jersey Commission on Cancer Research Fellowship.
1989-1992 Postdoctoral Fellowship (Leukemia Society of America)
1992-1993 Sokol Scholar (Whitehead Institute)
1992-1993 Ladies Auxiliary to the Veterans of Foreign Wars Postdoctoral Fellowship
1993-1995 Harcourt General Charitable Foundation Research Grant
1994 American Cancer Society (Massachusetts Division) Research Grant
1994-1998 U.S. Army Junior Faculty Breast Cancer Research Grant #DAMD17-94-J4258
1995-2001 American Cancer Society Research Grant
1995-2000 Scholar, Leukemia Society of America
1997-2002 NIH Research Grant 1R01GMCA55684-01

Publications (Partial listing)

Hinds, P.W., Finlay, C.A., Frey, A.B. and Levine, A.J. (1987). Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-*ras*-transformed cell lines. Mol. Cell Biol. 7: 2863-2869.

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Maren, M.C., Jost, C.A., Brooks, L., Irwin, M.S., O'Nions, J., Tidy, J.A., James, N., McGregor, J.M., Harwood, C.A., Yulug, I.G., Vousden, K.H., Allday, M.J., Gusterson, B., Ikawa, S., **Hinds, P.W.**, Crook, T. and Kaelin Jr., W.G.(2000). A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. Nature Genetics 25: 47-54.

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Thomas, D.M., Carty, S.A., Piscopo, D.M., Lee, J.-S., Wang, W.-F., Forrester, W.C., and **Hinds, P.W.** (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol. Cell 8: 303-316.

Piboonniyom S, Timmermann S, **Hinds P**, and Münger K. (2002). Aberrations in the MTS1 tumor suppressor locus in oral squamous cell carcinoma lines preferentially affect the INK4A gene and result in increased cdk6 activity. Oral Onc 38: 179-186..

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Principal Investigator/Program Director (Last, first, middle): Hinds, Philip W.

Revi ws

Tiemann, F., Musunuru, K., and **Hinds, P.W.** (1997). The Retinoblastoma Tumour Suppressor Protein and Cancer. In: *Protein Dysfunction in Human Genetic Disease*., Y. Edwards and D. Swallow, eds. Bios Scientific Publishers, Oxford. pp. 163-185.

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Todd R, **Hinds PW**, Munger K, Rustgi AK, Opitz OG, Suliman Y, Wong DT. (2002). Cell cycle dysregulation in oral cancer. Crit. Rev. Oral Bio. 13:51-6.

Research Support

Hinds, P.W.

ONGOING

1 R01 GM/CA55684 Hinds (PI) 5/01/1997-4/30/2002 30%
NIH GM/NCI $161,476
Proliferative effects of cdk4 and cdk6 dysregulation

The overall goal of this project is to identify the mechanisms by which cdk4 and cdk6 contribute to the tumorigenic phenotype with particular emphasis on discrete roles for these kinases in different cell types.

1 R01 AG/CA20208 Hinds (PI) 2/01/2002-1/31/2007 30%
NIH NIA/NCI $200,000
Role of pRb in osteogenesis, cell cycle exit and cancer

The goal of this project is to explore the mechanism through which pRb activates CBFA1-dependent transcription in particular and the role of pRb in bone differentiation in general. In addition, mechanisms of pRb action in cell cycle exit in senescence and differentiation are investigated.

1P01 DE12467 Wong (PI) 04/01/1998-03/31/2003 10%
NIH/NIDCR $131,308
Cell Cycle Regulators of Oral Cancer

The overall goal of this program project is to advance understanding of the molecular basis of oral cancer. Project one, "HPV and Cell Cycle Dysregulation in Oral Cancer", has as its goal the elucidation of the mechanisms of loss of cell cycle control in oral epithelial tumors. The budget is split equally with Karl Münger, who is co-PI of this project.

COMPLETED

RPG-95-013-04-CSM 01/01/1998-12/31/1999
American Cancer Society
Function of the Retinoblastoma Protein and D-Cyclins in Cancer

The goal of this project was to discriminate among specific roles of the retinoblastoma protein in cancer, differentiation and senescence. The role of cyclin D1 in development and cancer was an early goal of this project that is now the subject of ongoing work.

RPG-95-013-06-CCG Hinds (PI) 01/01/2000-12/31/2001
American Cancer Society $100,000
Function of pRb in Differentiation, Senescence and Cancer

The goal was to understand the role of pRb in terminal cell cycle exit at the cellular and organismal level.

[CANCER RESEARCH 54, 3662–3667, July 15, 1994]

Advances in Brief

Growth Suppression of Human Head and Neck Cancer Cells by the Introduction of a Wild-Type *p53* Gene via a Recombinant Adenovirus[1]

Ta-Jen Liu, Wei-Wei Zhang, Dorothy L. Taylor, Jack A. Roth, Helmuth Goepfert, and Gary L. Clayman[2]

Department of Head and Neck Surgery [T-J. L., D. L. T., H. G., G. L. C.], Section of Thoracic Molecular Oncology, and Department of Thoracic and Cardiovascular Surgery [W-W. Z., J. A. R.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Mutations of the *p53* gene constitute one of the most frequent genetic alterations in squamous cell carcinoma of the head and neck (SCCHN). In this study, we introduced wild-type *p53* into two separate SCCHN cell lines via a recombinant adenoviral vector, Ad5CMV-p53. Northern blotting showed that following infection by the wild-type *p53* adenovirus (Ad5CMV-p53), cells produced up to 10-fold higher levels of exogenous *p53* mRNA than cells treated with vector only (without *p53*). Western blotting showed that the increased levels of p53 protein produced in the Ad5CMV-p53-infected cells were a reflection of *p53* mRNA expression. *In vitro* growth assays revealed growth arrest following Ad5CMV-p53 infection as well as cell morphological changes consistent with apoptosis. *In vivo* studies in nude mice with established s.c. squamous carcinoma nodules showed that tumor volumes were significantly reduced in mice that received peritumoral infiltration of Ad5CMV-p53. These data suggest that Ad5CMV-p53 may be further developed as a potential novel therapeutic agent for SCCHN since introduction of wild-type p53 into SCCHN cell lines attenuates their replication and tumor growth.

Introduction

Patients with SCCHN[3] are afflicted with a disease process that often has profound effects on speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients has remained unchanged at approximately 45% for nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Treatment failures among these patients remain local and regional; only 10–15% of patients with the disease die of distant metastasis alone (2).

Although we have gained in understanding of the molecular events in the initiation and progression of SCCHN, they continue to require intensive investigation. A recent study identifying loss of heterozygosity of chromosome 9p21–22 as the most frequent genetic alteration in SCCHN suggested that this may be an early event in progression toward this neoplasm (3). Additionally, amplification and/or overexpression of cellular and nuclear oncogenes, such as c-*erB*-1 (4), *int*-2 (5), *bcl*-1 (6) and c-*myc* (7), have been documented in these cancers. The tum r suppressor gene *p53* has been the subject of immense interest and investigation in recent years. Alterations in the *p53* gene, including deletion, insertion, and point mutation, are the most frequent genetic events in many different carcinomas, such as those of the colon (8), breast (9), and lung (10), as well as soft-tissue sarcomas and leukemias (11). Several investigators have demonstrated the high frequency of *p53* gene alterations in SCCHN (12, 13).

There is considerable evidence implicating mutations of the *p53* gene in the etiology of many human cancers (14). Reports have demonstrated that growth of several different human cancer cell lines, including representatives of colon cancer (15), glioblastoma (16), breast cancer (17), and osteosarcoma (18), can be functionally suppressed by DNA transfection or retrovirus-mediated transfer f the wild-type *p53* gene. This gene may have an important role not only in cell growth but in apoptosis (programmed cell death). Induction of exogenous expression of wild-type *p53* has been shown to induce apoptosis in colon cancer cell lines (19) and in human lung cancer spheroids (20).

The adenoviral vector has emerged as a leading candidate for *in vivo* gene therapy in the past few years. It enjoys an advantage over traditional DNA transfection and retroviral transfer in its high efficiency of transferring potentially therapeutic genes into a wide range of host cells (21). The recently created adenoviral vector containing wild-type *p53* (Ad5CMV-p53; Ref. 22) provides us with an attractive delivery system to investigate the effect of exogenous wild-type *p53* on SCCHN cell lines both *in vitro* and *in vivo*. The outcome of this study indicates that further development of the p53 adenovirus or other novel molecular therapies for SCCHN is warranted.

Materials and Methods

Cell Lines and Culture Conditions. Human SCCHN cell lines Tu-138 and Tu-177 were both established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center. Tu-138 and Tu-177 were established from a gingivo-labial moderately differentiated squamous carcinoma and a poorly differentiated squamous carcinoma of the larynx, respectively. Both cell lines were developed via primary explant technique and are cytokeratin positive and tumorigenic in athymic nude and SCID mice. These cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FBS with penicillin/streptomycin.

Recombinant Adenovirus Preparation and Infection. The recombinant p53 adenovirus (Ad5CMV-p53; Ref. 22) contains the CMV promoter, wild-type p53 cDNA, and SV40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified Ad5. Viral stocks were propagated in 293 cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in phosphate-buffered saline, and lysed; cell debris was removed by subjecting the cells to CsCl gradient purification. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Infection was carried out by the addition of the virus to the DMEM/F12 medium and 2% FBS to the cell monolayers. The cells were incubated at 37°C for 60 min with constant agitation. Then complete medium (DMEM/F12–10% FBS) was added, and the cells were incubated at 37°C for the desired length of time.

Northern Blot Analysis. Total RNA was isolated by the acid-guanidinium thiocyanate method of Chomczynski and Sacchi (23). Northern analyses were performed on 20 μg of total RNA. The membrane was hybridized with a *p53*

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[1] This work was supported in part by: American Cancer Society Career Development Award 93–9 (to G. L. C.); M. D. Anderson Cancer Center Core Grant NIH-NCI-CA-16672; Grant R01 CA-45187 from the National Cancer Institute and Training Grant CA09611 (both to J. A. R.); by gifts to the Division of Surgery from Tenneco and Exxon for the Core Lab Facility; the University of Texas M. D. Anderson Cancer Center Core Grant CA16672; and by a generous gift from the Mathers Foundation.

[2] To whom requests for reprints should be addressed, at M. D. Anderson Cancer Center, Department of Head and Neck Surgery, Box 69, 1515 Holcombe Boulevard, Houston, TX 77030.

[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12 medium; FBS, fetal bovine serum; Ad5, adenovirus 5; CMV, cytomegalovirus; Ad5CMV-p53, wild-type p53 adenovirus; cDNA, complementary DNA; MOI, multiplicity of infection; SDS, sodium dodecyl sulfate; β-gal, β-galactosidase; dl312, replication-defective adenovirus; PFU, plaque forming units.

cDNA probe labeled by the random primer method in 5 × SSC-5 × Denhardt's solution-0.5% SDS-denatured salmon sperm DNA (20 μg/ml). The membrane was also stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA for RNA loading control. The relative quantities of *p53* expressed were determined by densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Western Blot Analysis. Total cell lysates were prepared by sonicating the cells 24-h postinfection in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) for 5 s. Fifty μg of protein from samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL membrane (Amersham). The membrane was blocked with Blotto/Tween (5% nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in phosphate-buffered saline) and probed with the primary antibodies, mouse anti-human p53 monoclonal antibody PAb1801 and mouse anti-human β-actin monoclonal antibody (Amersham), and the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN). The membrane was processed and developed as the manufacturer suggested.

Immunohistochemical Analysis. The infected cell monolayers were fixed with 3.8% formalin and treated with 3% H_2O_2 in methanol for 5 min. Immunohistochemical staining was performed by using the Vectastain Elite kit (Vector, Burlingame, CA). The primary antibody used was the anti-p53 antibody PAb1801, and the secondary antibody was an avidin-labeled anti-mouse IgG (Vector). The biotinylated horseradish peroxidase avidin-biotin complex reagent was used to detect the antigen-antibody complex. Preadsorption controls were used in each immunostaining experiment. The cells were then counterstained with Harris hematoxylin (Sigma Chemical Co., St. Louis, MO).

Cell Growth Assay. Cells were plated at a density of 2 × 10^4 cells/ml in 6-well plates in triplicate. Cells were infected with either wild-type (Ad5CMV-p53) or replication-deficient adenovirus as a control. Cells were harvested every 2 days and counted; their viability was determined by trypan blue exclusion.

Inhibition of Tumor Growth *in Vivo*. The effect of Ad5CMV-p53 on established s.c. tumor nodules was determined in nude mice in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and use and for recombinant DNA research. Briefly, following induction of acepromazine/ketamine anesthesia, three separate s.c. flaps were elevated on each animal, and 5 × 10^6 cells in 150 μl of complete media were injected s.c. into each flap using a blunt needle; the cells were kept in the pocket with a horizontal mattress suture. Four animals were used for each cell line. After 4 days, the animals were reanesthetized, and the flaps were reelevated for the delivery of 100 μl of: (*a*) Ad5CMV-p53 (10^8 PFU) in the right anterior flap; (*b*) replication-defective virus (10^8 PFU) in the right posterior flap; and (*c*) transport medium alone, in the left posterior flank. All injection sites had developed s.c. visual and palpable nodules before treatment was administered. Animals were observed daily and sacrificed on day 20. *In vivo* tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Following sacrifice, excised tumors were measured three dimensionally by microcalipers to determine tumor volume. A nonparametric Friedman's two-way analysis of variance test was used to test the significance of the difference between means of samples; the SPSS/PC+ software package (SPSS, Inc., Chicago, IL) was used.

Results

Aden viral Infection of SCCHN Cells. The conditions for optimal adenoviral transduction of Tu-138 and Tu-177 cells were determined by infecting these cells with adenovirus expressing the *Escherichia coli* β-gal gene. The transduction efficiency was assessed by counting the number of blue cells after X-gal staining. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used. Cells inoculated with a single dose of 100 MOI β-gal adenovirus exhibited 60% blue cells (Fig. 1*A*), and this was improved t 100% by multiple infections (data not shown). The transduction efficiency of this vector in SCCHN cells is quite different from that of other cell lines examined previously; HeLa, HepG2, LM2, and human non-small cell lung cancer cell lines

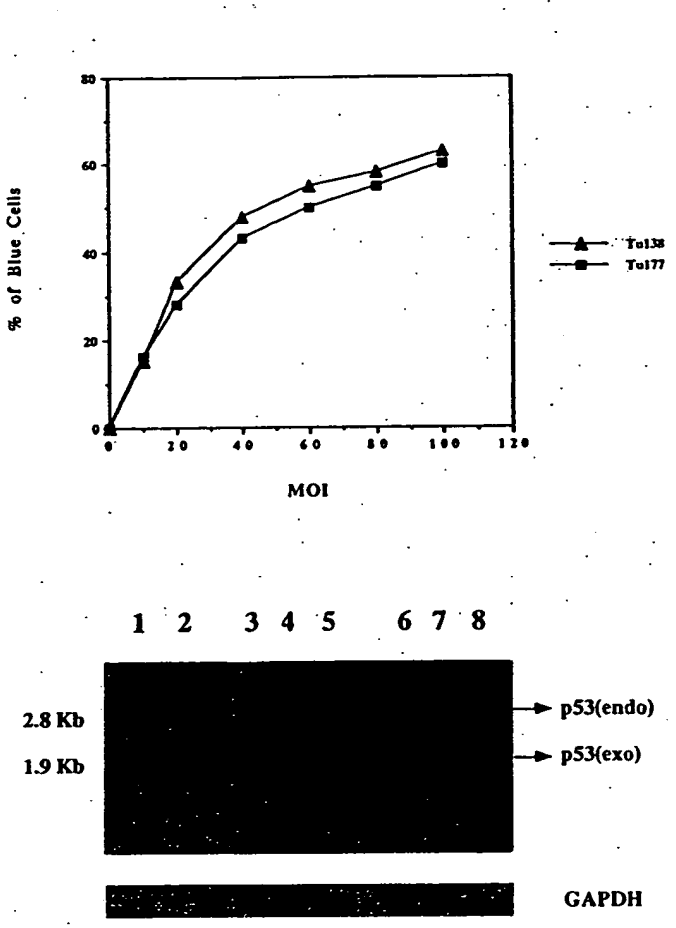

Fig. 1. *A*, transduction efficiency of SCCHN cell lines Tu-138 (▲) and Tu177 (■). A recombinant β-gal adenovirus was used to infect the cells at different MOIs ranging from 10 to 100. The percentages of β-gal-positive cells were obtained from scoring 500 cells each on replicate dishes. *B*, expression of exogenous *p53* mRNA 24 h after Ad5CMV-p53 infection. *Lanes 1* and *2*, 293 and K562 cells, respectively. *Lanes 3* and *6*, mock-infected Tu-138 and Tu-177 cells. *Lanes 4* and *7*, Tu-138 and Tu-177 cells infected with dl312. *Lanes 5* and *8*, Tu-138 and Tu-177 cells infected with Ad5CMV-p53.

showed 97 to 100% infection efficiencies after incubation with 30 to 50 MOI β-gal adenovirus (22).

Expression of Exogenous *p53* mRNA in Adenovirus-infected SCCHN Cells. Two human SCCHN cell lines were chosen for this study; both cell lines Tu-138 and Tu-177 possess a mutated *p53* gene (unpublished data). The recently created recombinant wild-type p53 adenovirus, Ad5CMV-p53, was used to infect Tu-138 and Tu-177 cells. Twenty-four h after infection, total RNA was isolated, and Northern blot analysis was performed. The transformed primary human embryonal kidney cell line 293 was used as a positive control because of its high level of expression of the *p53* gene product, whereas K562, a lymphoblastoma cell line with a homozygous deletion of the *p53* gene, was the negative control (Fig. 1*B*, *Lanes 1* and *2*, respectively). Due to unequal loading, only a fraction of the endogenous *p53* mRNA was detected in the 293 cells (Fig. 1*B*, *bottom panel*). The levels of the 2.8-kilobase endogenous *p53* mRNA detected in the samples isolated from mock-infected cells (Fig. 1*B*, *Lanes 3* and *6*) and from the cells infected with a replication-defective adenovirus, dl312 (Fig. 1*B*, *Lanes 4* and *7*), were similar. Up to 10-fold higher levels f ex genous 1.9-kilobase *p53* mRNA were present in the cells infected with Ad5CMV-p53 (Fig. 1*B*, *Lanes 5* and *8*), indicating that the exogenous *p53* cDNA was successfully transduced into these cells and efficiently transcribed. Interestingly, the level of endogenous *p53* mRNA in these cells was 5-fold higher than in the experimental controls. Northern blots exhibited no evidence of Ad5CMV-p53 (DNA) contamination of RNA.

Expression of p53 Protein in Aden virus-infected SCCHN Cells. Western blot analysis was performed to compare the levels of *p53* mRNA t the amount of p53 protein produced. A p53 band, recognized by monospecific anti-p53 antibody, PAb1801, was observed in cellular extracts isolated from all samples except K562 cells (Fig. 2*A*, *Lane 8*). Cell line 293 showed high levels of p53 protein (Fig. 2*A*, *Lane 1*). Samples isolated from mock-infected Tu-138 and Tu-177 cells exhibited low levels of p53 protein (Fig. 2*A*, *Lanes 2* and *5*). The level of p53 expression remained similar in those cells infected with the dl312 adenovirus (Fig. 2*A*, *Lanes 3* and *6*). The levels of p53 antigen detected in Ad5CMV-p53-infected cells were significantly higher than the levels of the endogenous mutated proteins in both cell lines (Fig. 2*A*, *Lanes 5* and *7*). This result indicates that the exogenous *p53* mRNA produced from cells infected with Ad5CMV-p53 is efficiently translated into immunoreactive p53 protein. Furthermore, immunohistochemical analysis of cells infected with Ad5CMV-p53 revealed the characteristic nuclear staining of p53 protein (Fig. 2*B*, *right panel*), whereas mock-infected cells failed to show similar staining despite the presence of the p53 protein in these cells (Fig. 2*B*, *left panel*). This inability to detect the protein may be attributable to the insensitivity of the assay.

Effect of Exogenous p53 on SCCHN Cell Growth *in Vitro*. Cells infected with control virus dl312 had growth rates similar to those of the mock-infected cells (Fig. 3), whereas growth of the Ad5CMV-

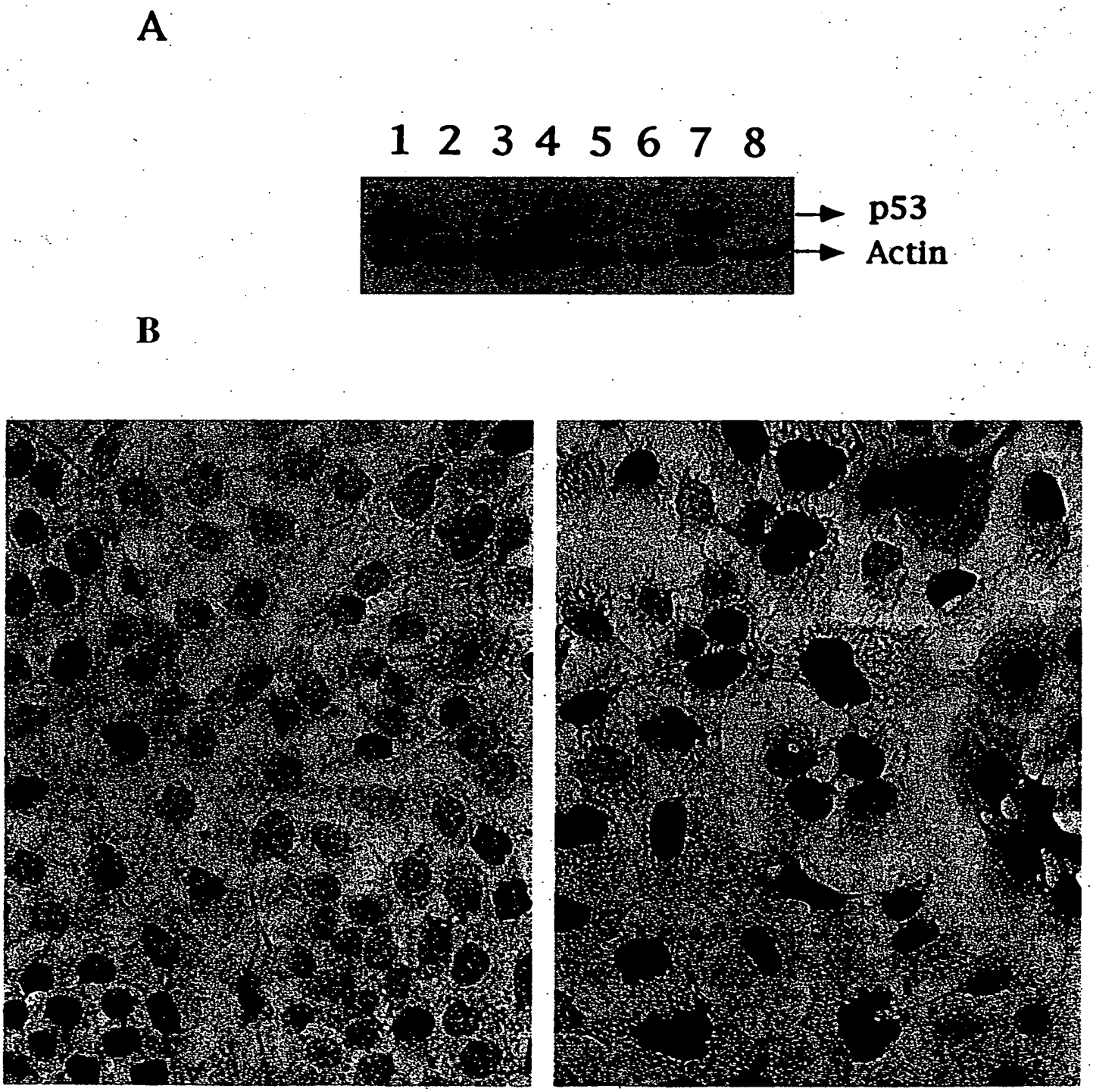

Fig. 2. *A*, Western blot analysis. Cellular extracts isolated from cells 24 h postinfection were subjected to SDS-polyacrylamide gel electrophoresis. *Lanes 1* and *8*, 293 and K562 cells, respectively. *Lanes 2* and *5*, mock-infected Tu-138 and Tu-177 cells. *Lanes 3* and *6*, Tu-138 and Tu-177 cells infected with dl312. *Lanes 4* and *7*, Tu-138 and Tu-177 cells infected with the Ad5CMV-p53. *B*, representative immunohistochemical staining of mock-infected Tu-138 cells (*left*) and Ad5CMV-p53-infected Tu-138 cells (*right*) 24-h postinfection. × 250.

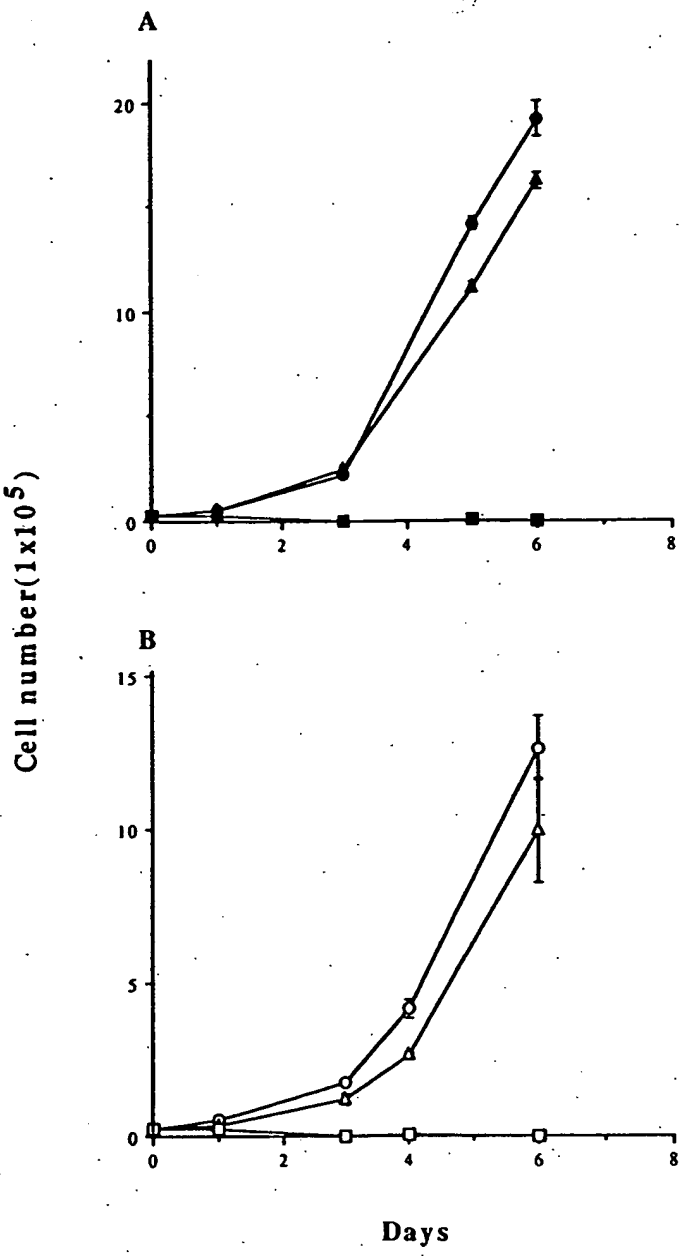

Fig. 3. Inhibition of SCCHN cell growth *in vitro*. *A*, growth curve of mock-infected Tu-138 cells (●), dl312-infected cells (▲), and Ad5CMV-p53-infected cells (■). *B*, growth curve of mock-infected Tu-177 cells (○), dl312-infected cells (△), and Ad5CMV-p53-infected cells (□). At each indicated time point, three dishes of cells were trypsinized and counted. The mean of cell counts per triplicate wells following infection were plotted against the number of days since infection; *bars*, SEM.

p53-infected Tu-138 (Fig. 3*A*) and Tu-177 (Fig. 3*B*) cells was greatly suppressed. Twenty-four h after infection, an apparent morphological change occurred with portions of the cell population rounding up and their outer membranes forming blebs. These are part of a series of histologically predictable events that constitute programmed cell death. The effect was more prominent for Tu-138 than for Tu-177 cells. Cells infected with the replication-defective adenovirus, dl312, dem nstrated normal growth characteristics with no histomorphological abnormalities. Growth assays were reproducible in four repeated experiments.

Inhibition of Tum r Growth *in Vivo*. Seven animals were tested for each cell line. One animal in the Tu-177 group died following the second flap surgery and delivery of the therapeutic interventions, presumably due to profound anesthesia and subsequent mutilation by cage mates. Necropsy revealed no evidence of metastasis or systemic effects. Fig. 4 shows representative Tu-138 (*left*) and Tu-177 recipients (*right*). Sizable tumors are apparent on both posterior flaps of the animals (*i.e.*, the sites that did not receive Ad5CMV-p53). The lack of tumor progression is significant in the right anterior flaps of the animals which received Ad5CMV-p53 ($P < .04$). That Tu-177 cells have a slower growth rate has been established previously in these animals.[4] Two animals in the Tu-177 group had complete clinical and pathological regression of their established s.c. tumor nodule. Tw animals in the Tu-138 group were killed early because they were experiencing rapid growth and ulceration of the control tumor sites. All surgical sites had developed lesions of at least 6 mm^3 before intervention. The tumor volumes on necropsy are shown in Table 1.

Discussion

Mutations or deletions of the *p53* tumor suppressor gene are the most frequent genetic alterations reported in SCCHN. Since the wild-type *p53* gene is believed to be involved primarily in delivering antiproliferative signals that may be capable of antagonizing the growth-stimulatory signals propagated by oncogene products, the potential molecular therapeutic effect of this gene in SCCHN deserves attention.

The rapid development in the field of gene therapy, including the creation of adenoviral vectors, has created an environment that is well suited for progress toward novel gene therapy of SCCHN. Because of their natural tropism for aerodigestive tract epithelium, adenoviruses may be uniquely suitable for the transient delivery of genes to cancers in these epithelial tissues. The recombinant, replication-defective adenoviruses that have been developed for gene therapy are missing the entire E1a and part of the E1b regions and are, therefore, capable of propagating only in cells that can provide the E1 proteins in trans, such as the 293 cell line. In the past few years, recombinant adenoviruses have been extensively developed and used for *in vivo* gene therapy. The high transfer efficiency of adenoviral vectors over a broad range of hosts both *in vitro* and *in vivo* make them attractive vehicles for molecular therapy. Recently, a recombinant wild-type p53 adenoviral vector (Ad5CMV-p53) was generated. This provided us with an excellent candidate for investigation of the biological effects of the wild-type *p53* gene product on SCCHN cells bearing the mutated *p53* gene. Using a β-gal recombinant adenovirus, the gene transfer efficiency of SCCHN cells was established. Approximately 60% of SCCHN cells were positive after X-gal staining. There appeared to be a linear correlation between the number of cells expressing the gene and the amount of viral particles used in the experiment. This result coincided with the efficiency obtained in cells infected with Ad5CMV-p53 after immunostaining by using a monoclonal anti-p53 antibody. Our observed transduction efficiency was lower than that achieved in other cell lines tested, including HeLa, HepG2, LM2, and the human non-small cell lung cancer cell lines. This discrepancy could be due to a host of factors, including receptor variations and differences in membrane characteristics among the cell lines. Additionally, the transduction efficiency of SCCHN cells may have been underestimated by limitations of light microscopic analyses.

Ad5CMV-p53 mediated a high level of expression of the *p53* gene in SCCHN cells. Two *p53* mRNA species were detected in the Ad5CMV-p53-infected cells. The high level of 1.9-kilobase mRNA was derived from the transduced *p53* cDNA following infection with Ad5CMV-p53, indicating that the adenoviral vector is an efficient vehicle for gene delivery into human SCCHN cells. Moreover, the levels of endogenous 2.8-kilobase mRNA were higher in the transduced cells than in the controls, presumably due to the effect f wild-type *p53* gene product. This phenomenon of transcriptional

[4] Unpublished data.

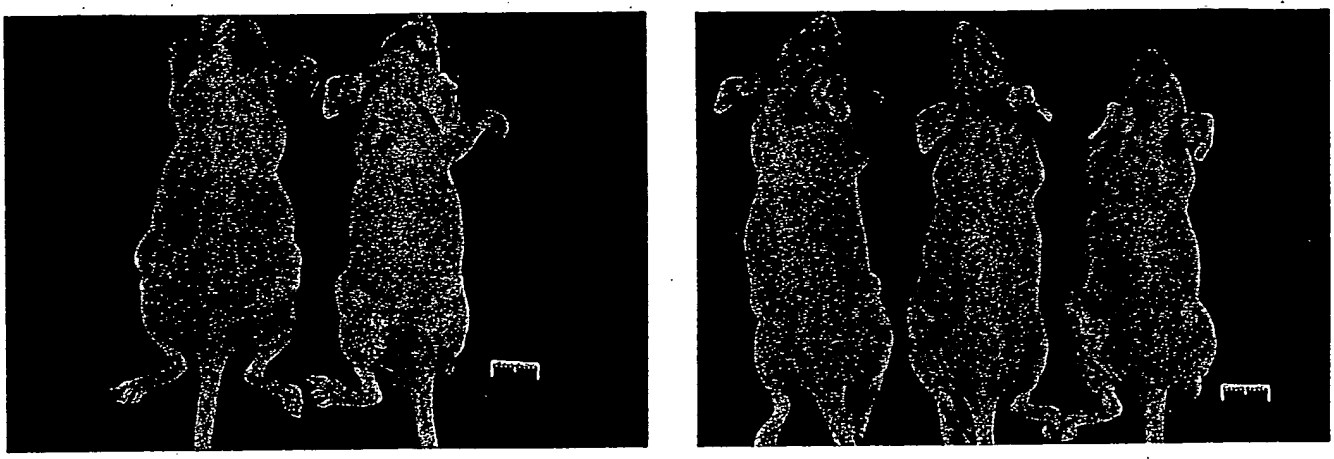

Fig. 4. Inhibition of SCCHN cell growth *in vivo*. Pictures of the representative nude mice studies for both Tu-138 (*left*) and Tu-177 (*right*) cell lines 20 days following therapeutic interventions. The right posterior flank received dl312, the left flank received transport medium alone, and the right anterior flap received Ad5CMV-p53, all 4 days following the establishment of a s.c. tumor.

Table 1 *Effect of Ad5CMV-p53 on tumor growth in nude mice*[a]

Treatment	Mean volume (mm^3 ± SEM) Tu-138 (7)	Tu-177 (6)
Ad5CMV-p53	22.3 ± 14	13 ± 18
Ad5(dl312)	803 ± 300	533 ± 148
Medium	1297 ± 511	421 ± 143
Significance	*P*	*P*
p53[b]:dl312	0.03	0.02
p53:medium	0.04	0.03

[a] The cells were injected s.c. at 5×10^6 cells/flap. Tumor sizes were determined at day 20 after treatment. Numbers in parentheses, the number of animals evaluated.
[b] Ad5CMV-p53 is abbreviated as p53; dl312 is an abbreviation for Ad5(dl312).

autoregulation of the *p53* gene has been well documented in murine cell lines in which the wild-type *p53* can transactivate its own promoter and the mutant *p53* fails to regulate the *p53* promoter (24).

Due t the episomal property of adenoviral vectors, all the input DNA following infection with Ad5CMV-p53 is presumably degraded slowly throughout incubation. By using polymerase chain reaction-based detection techniques, DNA can be detected as late as 14 days postinfection (data not shown).

Western blot analysis demonstrated that there were few or no changes of p53 protein levels between mock- and replication-defective adenovirus-infected cells, whereas production of p53 protein was significant in Ad5CMV-p53-infected cells, suggesting that the exogenous *p53* mRNA was efficiently translated. Time course protein expression studies have shown protein expression to peak 3 days postinfection and progressively decline to still detectable Western blotting levels on day 15 (22). Functionally, these SCCHN cells transduced with wild-type p53 gene exhibited significant inhibition of growth *in vitro* as compared to the mock-infected and replication-defective cells, thus clearly illustrating that these results were not mediated by the virus itself. The mechanism by which wild-type p53 protein inhibits growth *in vitro* may be related to arrest f the G_1 cell cycle (18), apoptosis (19, 20), or induction of another tumor suppressor gene such as *WAF1/CIP1* (25). The induction of apoptosis is one of the several documented functions of wild-type *p53*. When Tu-138 and Tu-177 cells were infected with Ad5CMV-p53 at 100 plaque-forming units/cell, the characteristic apoptotic histomorphology, such as rounded-up cells and the formation of blebs, was apparent as early as 4 h after infection and was followed rapidly by cell death (data not shown). However, the mechanism of growth suppression and cell death induced by Ad5CMV-p53 requires further investigation.

Encouraging results were also obtained in the nude mice studies. Tumor growth in the Ad5CMV-p53-infected cells was suppressed by at least 60 times more than in the experimental controls. These *in vivo* results confirmed the *in vitro* effects of Ad5CMV-p53 on human SCCHN cells, suggesting that the wild-type p53 protein mediates a potentially therapeutic effect. Although the *in vivo* studies are in their infancy, they nevertheless portend the development of a model for gene therapy in SCCHN that uses p53 adenovirus as a therapeutic intervention. Information derived from such studies could be expanded in the development of other novel molecular therapies that use adenoviral vectors, not only in SCCHN but in other human cancers. Several critical questions remain unanswered. How should the insult from antibodies that may arise in animals or patients following viral treatment be alleviated? How safe is this virus in humans? The results of the preliminary studies justify further investigation of *in vivo* animal models as well as mechanisms through which wild-type *p53* regulates these *in vitro* and *in vivo* effects.

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Genetic Mechanisms of Tumor Suppression by the Human p53 Gene

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Mutations of the gene encoding p53, a 53-kilodalton cellular protein, are found frequently in human tumor cells, suggesting a crucial role for this gene in human oncogenesis. To model the stepwise mutation or loss of both p53 alleles during tumorigenesis, a human osteosarcoma cell line, Saos-2, was used that completely lacked endogenous p53. Single copies of exogenous p53 genes were then introduced by infecting cells with recombinant retroviruses containing either point-mutated or wild-type versions of the p53 cDNA sequence. Expression of wild-type p53 suppressed the neoplastic phenotype of Saos-2 cells, whereas expression of mutated p53 conferred a limited growth advantage to cells in the absence of wild-type p53. Wild-type p53 was phenotypically dominant to mutated p53 in a two-allele configuration. These results suggest that, as with the retinoblastoma gene, mutation of both alleles of the p53 gene is essential for its role in oncogenesis.

TUMOR-SUPPRESSOR GENES ARE DEfined as genes for which loss-of-function mutations are oncogenic (*1*). Wild-type alleles of such genes may thus function to prevent or suppress tumorigenesis. For example, introduction of wild-type copies of the retinoblastoma gene (*RB*), the prototype of this class (*2*), suppressed the neoplastic properties of human tumor cells with mutated endogenous RB, thereby providing direct evidence for tumor suppression by a single gene (*3, 4*). Another gene product, p53, was first identified as a 53-kD cellular protein that binds to SV40 T antigen (*5*), a property that is also shared by RB protein. The gene encoding p53 is commonly affected by deletions, rearrangements, or point mutations in human and murine tumor cells (*6, 7*). p53 was originally considered to be an oncogene because mutated p53 alleles could transform primary rat embryo fibroblasts in concert with an activated *ras* gene (*8*). However, cotransfection of wild-type murine p53 was shown to reduce transformation efficiency by many other oncogenes (*9*). These studies, and the observed diversity of mutations in human tumors, suggested that p53 might be a tumor suppressor gene, that is, a gene that is inactivated by mutation. The dominant transforming effect was presumed to be due to a "dominant negative" activity of mutated p53 protein that somehow blocked the growth-restricting function of wild-type p53 protein in cells. This model suggested that the relative quantity of mutated to wild-type p53 could determine the transformed phenotype, but gene dosages could not be tightly controlled in these transfection studies.

Because of such questions, as well as the possibility of species-specific differences in p53 function (*10*) and the uncertain relevance of transformed animal cells to human neoplasia, we sought to reassess the biological properties of p53 in the human system. The human osteosarcoma cell line Saos-2 was chosen as a host cell because it has no endogenous p53, because of the complete deletion of its gene (*6*). We used recombinant retroviruses derived from Moloney murine leukemia virus (Mo-MuLV) to introduce mutated or wild-type p53 under the long terminal repeat (LTR) promoter control. Cell clones isolated after infection and selection carried only a single integrated provirus of each type, and multiple clones were analyzed to exclude positional effects. A comprehensive assessment of biological properties of these clones included morphology, growth rates and saturation density in culture, colony formation in soft agar, and tumorigenicity in nude mice.

As a reference standard for human wild-type p53, we used the genomic DNA sequence of Lamb and Crawford (*11*). Poten-

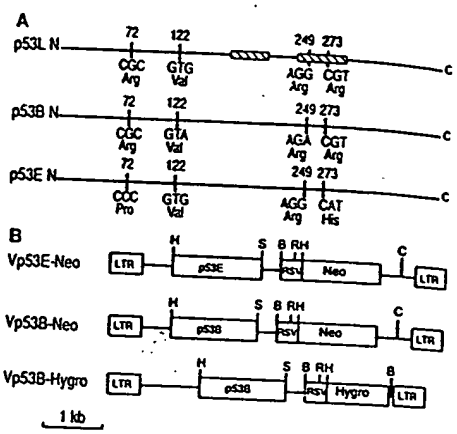

Fig. 1. Comparison of three human p53 cDNAs, and genomic organization of three recombinant retroviruses for expressing p53 protein. (A) Three human p53 cDNAs are diagrammed. The sequence reported by Lamb and Crawford (*11*), here labeled as p53L, was derived by sequencing clones from human fetal liver cDNA and genomic libraries, and is considered to be wild type. p53B is a cDNA clone derived from fetal brain RNA by the RT-PCR method (*12*). The deduced amino acid sequences of p53B and p53L were identical despite two silent nucleotide substitutions as indicated. p53E is a cDNA clone (*13*) that has amino acid substitutions at positions 72 and 273 relative to p53L or p53B. The Arg/Pro^{72} replacement represents a common amino acid polymorphism (*15*) without known functional significance, but the substitution of His for Arg at position 273 is found exclusively in tumor cells and is considered to be a mutation. Like many other p53 mutations, Arg^{273} → His lies within one of two conserved regions required for binding to SV40 T antigen (hatched boxes) (*23*). (B) Genomic organization of three p53 retroviruses are diagrammed. Vp53E-Neo was constructed by inserting a 1.5-kb Hind III–Sma I DNA fragment containing p53E into the plasmid pLRbRNL (*3*), replacing *RB* cDNA. A 1.35-kb p53B DNA fragment obtained by RT-PCR was inserted into the pLRbRNL vector to form Vp53B-Neo. The insert in one clone was entirely sequenced, as diagrammed in (A). Vp53B-Hygro was constructed by insertion of a Hind III DNA fragment containing p53B and the Rous sarcoma virus promoter into plasmid 477 (a MuLV-Hygro vector provided by W. Hammerschmidt and B. Sugden). These constructs were then used to produce the corresponding viral stocks as described previously (*3*). Some major restriction sites important for construction are indicated. H, Hind III; R, EcoR I; S, Sma I; B, Bam HI; and C, Cla I.

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cially wild-type p53 cDNA was isolated from fetal brain RNA by the method of reverse transcription–polymerase chain reaction (RT-PCR), and was cloned into plasmid (*12*). The insert in one clone (designated p53B) was entirely sequenced (~1300 bp) to reveal a wild-type deduced amino acid sequence despite two silent nucleotide replacements (Fig. 1A). Another p53 cDNA clone (p53E), isolated from epidermoid carcinoma cell line A431 (*13*), was also sequenced, and was found to contain a point mutation at codon 273 that replaced Arg with His (Fig. 1A). This is a functionally significant mutation that has also been identified in p53 from two other tumor cell lines (*14*). In addition, a neutral sequence polymorphism in codon 72 (Fig. 1A) encoded either an Arg (p53B) or a Pro (p53E). This common amino acid polymorphism (*15*), which is without known functional significance, resulted in faster migration of p53B than p53E protein by SDS–polyacrylamide gel electrophoresis (PAGE), and was therefore used to distinguish between these proteins when they were coexpressed in the same cell.

The p53E and p53B fragments were then inserted into a Mo-MuLV–based retroviral vector containing *neo* as a selectable marker gene to form Vp53E-Neo and Vp53B-Neo viral genomes, respectively (Fig. 1B). In addition, to facilitate double replacement, Vp53B-Hygro was made by inserting p53B into a similar vector containing the gene conferring resistance to hygromycin (*16*). Stocks of Vp53E-Neo, Vp53B-Neo, and Vp53B-Hygro viruses were produced as described (*3*) with titers of about 1×10^5, 2×10^4, and 1×10^5, respectively. Expression of p53 proteins from the viruses was initially assessed in the murine NIH 3T3–derived packaging line PA12, which was used for virus production (*3*). Mutated and wild-type human p53 proteins were detected in their respective virus-producing cells, with the expected difference in migration by SDS-PAGE (*17*). Because spontaneous mutation of p53 may occur frequently in cultured cells, we examined two additional biochemical properties of these p53 proteins: their cellular half-lives, and their ability to bind to T antigen. The p53B protein had a half-life of 20 to 30 min compared to 4 to 5 hours for p53E protein (*17*), consistent with published reports on the half-lives of wild-type and mutated p53 proteins (*18*). When virus-producing cells were transfected with a plasmid expresssing large amounts of SV40 T antigen, and lysates were immunoprecipitated with antibody to p53 (anti-p53) or antibody to T antigen (anti-T), T antigen was coprecipitated with p53B but not p53E protein (*17*), indicating that only p53B protein could bind to T. These results suggested that p53B-containing viruses expressed wild-type p53 and that p53E-containing virus expressed mutated p53 (*19*).

Fig. 2. Expression of human p53 proteins in virus-infected Saos-2 cells. Saos-2 cells (lanes 1 and 7) were infected either with (A) Vp53E-Neo to generate p53EN (lanes 2 to 6) or with (B) Vp53B-Neo and Vp53B-Hygro to generate p53BN (lanes 8 to 10) and p53BH (lanes 11 and 12) clones, respectively, as described in the text. (C) Saos-2 cells were also doubly infected with Vp53E-Neo and Vp53B-Hygro to generate p53EN-BN clones (lanes 13 to 15). Randomly selected clones, and WERI-Rb27 cells (lanes M), were labeled with [^{35}S]methionine and immunoprecipitated with anti-p53 antibody, PAb421 (*24*) as described for (*25*). p53B (filled arrows) and p53E (open arrows) are indicated.

In previous experiments, Saos-2 cells infected with parental viruses containing only neomycin- or hygromycin-resistance genes showed no changes in morphology and growth rate compared to uninfected cells (*3, 17*), suggesting that drug selection did not have a significant influence on their neoplastic properties. Saos-2 cells infected with comparable titers of either Vp53E-Neo, Vp53B-Neo, or Vp53B-Hygro in the presence of the appropriate selective agent each yielded similar numbers of drug-resistant colonies. Most colonies could be individually propagated into mass cultures, with the notable observation that Vp53B-infected cells grew much more slowly than Vp53E-infected cells (see below). Vp53E-infected clones uniformly expressed high concentrations of p53E protein (Fig. 2A). Of 30 Vp53B-infected clones examined, about 80% expressed detectable p53B protein (Fig. 2B). Two each of Vp53E-Neo and

Table 1. Neoplastic properties of p53 virus-infected Saos-2 cells. Soft-agar colony formation: equal numbers (1×10^5 or 2.5×10^4) of cells of the indicated clones were seeded in duplicate in 0.367% soft agar as described (*3*). Total colony numbers were scored after 20 days. Individual colonies contained more than 50 cells. Tumorigenicity: 1×10^7 cells from each clone were injected subcutaneously into flanks of nude mice, and tumor formation was scored at 12 weeks.

Virus-infected cells	No. of soft-agar colonies formed with		Tumorigenicity	p53 expression
	1.0×10^5 cells seeded	2.5×10^4 cells seeded	No. of mice with tumor/no. of mice injected	
Parental	392;388	104;76	10;10	None
p53EN			12;12	Mutated
p53EN-1	928;968	396;372		
p53EN-2	517;593	121;105		
p53EN-3	485;534	96;123		
p53EN-4	445;498	106;121		
p53EN-5	582;441	132;172		
p53BN			0;5	Wild type
p53BN-1	<1;<1	<1;<1		
p53BN-2	<1;<1	<1;<1		
p53BN-3	<1;<1	<1;<1		
p53BN-4	<1;<1	<1;<1		
p53BN-R	414;384	54;48	3;3	None
p53BH			0;6	Wild type
p53BH-1	<1;<1	<1;<1		
p53BH-2	<1;<1	<1;<1		
p53BH-3	<1;<1	<1;<1		
p53EN-BH			0;5	Mutated + wild type
p53EN-1-BH-1	<1;<1	<1;<1		
p53EN-1-BH-2	<1;<1	<1;<1		
p53EN-1-BH-3	<1;<1	<1;<1		
p53EN-2-BH-1	<1;<1	<1;<1		
p53EN-2-BH-2	<1;<1	<1;<1		

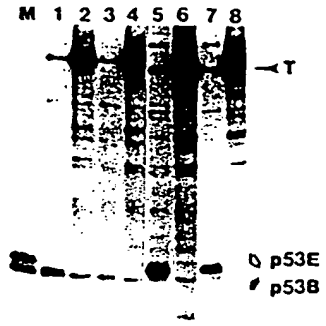

Fig. 3. Formation of p53B-T complexes in Saos-2 cells. Clones p53BN-1 (lanes 1 and 2), p53BH-1 (lanes 3 and 4), p53EN-1-BH-1 (lanes 5 and 6), and p53EN-1-BH-2 (lanes 7 and 8) were transfected with plasmid pRSV40T as described (*25*), and 60 hours later were metabolically labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with PAb421 (lanes M, 1, 3, 5 and 7) or with PAb419, a monoclonal antibody against SV40 T antigen (lanes 2, 4, 6 and 8). PAb419 coprecipitated only p53B in cells expressing both p53B and p53E.

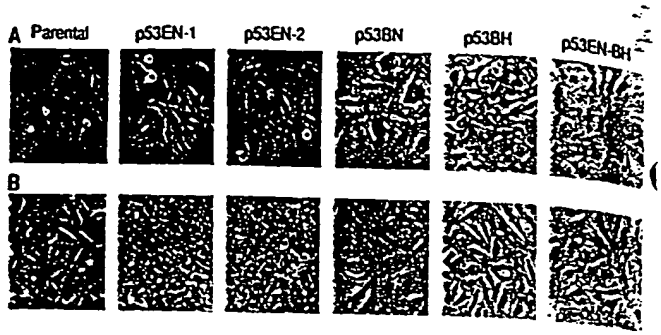

Fig. 4. Morphology in culture of parental Saos-2 cells, and representative virus-infected clones. (A) Exponentially growing cells. (B) Cells at confluency. Magnification, ×100.

Vp53B-Hygro clones were randomly selected for a second infection by the other virus, and double-infected clones were isolated and propagated as above. These clones coexpressed both p53E and p53B protein (Fig. 2C). In order to again verify that p53B protein in these cells was not secondarily mutated, p53B-expressing clones were transfected with the SV40 T antigen plasmid, and cell lysates were immunoprecipitated as described above (Fig. 3). Anti-p53 coprecipitated T in each clone, but anti-T coprecipitated only p53B, even in cells expressing both p53B and p53E. The half-life of p53B in Saos-2 was also measured and was similar to that of p53B in PA12 cells (*17*). These data again support the notion that Vp53B-infected Saos-2 clones expressed wild-type p53.

Five randomly chosen clones that stably expressed p53E protein (p53EN-1 to p53EN-5) were compared to parental Saos-2 cells in terms of morphology (Fig. 4), growth rate [as doubling time (Fig. 5A)], saturation density (Fig. 5B), soft-agar colony formation, and tumorigenicity in nude mice (Table 1). A difference in morphology was observed only under conditions of cell crowding, where cells of EN clones were far smaller and more refractile than parental cells (Fig. 4B). Correlatively, saturation density of the former was four- to fivefold greater than that of parental cells (Fig. 5B). This relative growth advantage was seen despite similar doubling times as measured under sparse growth conditions (Fig. 5A). Four EN clones and parental cells shared similar efficiencies in soft-agar colony formation and tumorigenicity in nude mice (Table 1). One clone, p53EN-1, had noticeably augmented abilities in both respects; in particular, it reliably formed large tumors from as few as 5×10^5 injected cells (*17*). We considered this discrepancy to be within the range of clonal variability expected among tumor cells. These results suggested that mutated p53 functioned in the absence of wild-type p53 to confer a limited growth advantage (higher saturation density) to Saos-2 cells in culture. In many other aspects of the neoplastic phenotype, the presence of point-mutated p53 was essentially equivalent to complete absence of p53.

In comparison to parental Saos-2 cells, clones expressing p53B (wild-type p53 protein) were invariably enlarged and flattened (Fig. 4) and had prolonged doubling times in culture of about 70 hours rather than 30 to 36 hours for parental or EN cells (Fig. 5A). The efficiency of soft-agar colony formation was reduced to less than the threshold for detecting a single colony, whereas parental cells and EN cells formed hundreds of colonies under the same conditions (Table 1). Injection of 1×10^7 cells of each of seven p53B-expressing clones into the flanks of nude mice resulted in the formation of no tumors after 12 weeks, even while the same number of parental or p53E-expressing cells formed tumors in all contralateral flanks (Table 1). These findings could not be explained by a peculiar effect of viral infection and selection because one clone, Vp53BN-R, derived from Vp53B-Neo–infected cells but lacking detectable expression of p53B, had a phenotype indistinguishable from parental cells (Table 1). The ~50% reduction of growth rate of cultured Saos-2 cells expressing p53B was insufficient to account for the complete loss of tumorigenicity and soft-agar colony formation, implying that wild-type p53 specifically suppressed the neoplastic phenotype of these cells. These results suggest that loss of wild-type p53 was a significant event during the genesis of this tumor line, and, by extension, of other osteosarcomas with mutated endogenous p53 genes (*6*).

Because both mutated and wild-type p53 proteins were apparently functional in Saos-2 cells, we asked whether both activities could be simultaneously coexpressed, whether they canceled out one another, or whether one activity was clearly dominant. The configuration of one wild-type and one mutated allele was most relevant to natural human tumorigenesis, because this is a necessary intermediate step on the pathway toward complete loss of wild-type p53. Infection of two different p53E-expressing clones with Vp53B-Hygro yielded 22 hygromycin-resistant clones, of which 15 coexpressed both p53B and p53E. To determine the number of integrated copies of each virus present in these clones, we analyzed genomic DNA of three clones derived from p53EN-1 cells infected with Vp53B-Hygro by Southern (DNA) blotting (Fig. 6). Hybridization with *neo* as a probe showed a single, common junctional frag-

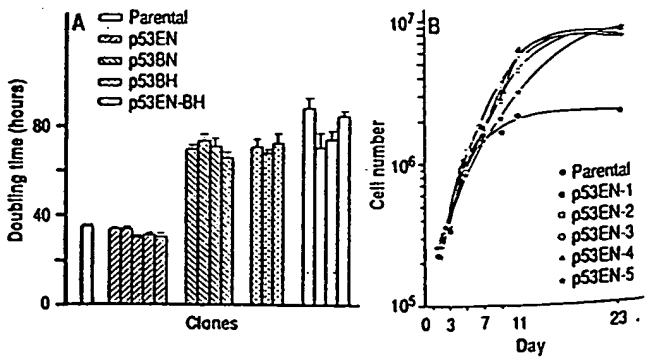

Fig. 5. Growth effects of p53 expression in Saos-2 cells. (A) Doubling times of parental Saos-2 cells and virus-infected clones in an exponential growth stage. Equal numbers of each cell type were seeded into 60-mm culture dishes; cells of two dishes were trypsinized and counted at daily intervals for 4 days. Doubling times were derived from lines fitted to log cell numbers. (B) Saturation density of parental Saos-2 and EN clones. Equal numbers (1×10^5) of cells were seeded into 60-mm culture dishes; cells of two dishes were trypsinized and counted at the times indicated. Plotted points were mean cell numbers from duplicate dishes. Saturation density of p53E-expressing cells was four- to fivefold greater than parental cells.

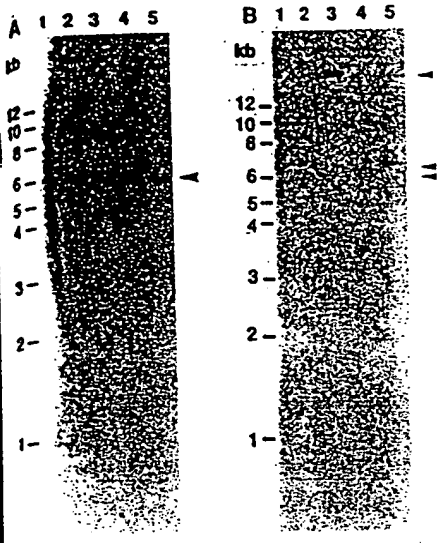

Fig. 6. p53EN-BH cells harbored one copy of Vp53E-Neo and one copy of Vp53B-Hygro. Genomic DNA (10 μg) extracted from parental Saos-2 cells (lanes 1), and clones p53EN-1 (lanes 2), p53EN-1-BH-1 (lanes 3), p53EN-1-BH-2 (lanes 4), and p53EN-1-BH-3 (lanes 5), was digested with Eco RI, and separated in 0.7% agarose gels. Southern transfer was performed, and nylon membranes were hybridized with ^{32}P-labeled *neo* (A) or *hygro* (B) DNA probes, by standard methods (*26*). A single, unique junction fragment is seen in each clone with each probe, indicating single integrated copies of each virus.

ment in all three clones, indicating the presence of a single integrated copy of Vp53E-Neo in p53EN-1 cells (Fig. 6A). Hybridization with *hygro* showed a single, unique junctional fragment in each clone, indicating the presence of single, independently integrated copies of Vp53B-Hygro in p53EN-BH clones (Fig. 6B). Single integrations were expected, based on previous use of a related recombinant retrovirus at comparable titers (*3*). These findings confirmed that p53EN-BH clones indeed contained one integrated copy of each virus, and that both exogenous p53 genes were expressed (Fig. 2). By criteria of morphology, growth rate, saturation density, soft-agar colony formation, and tumorigenicity in nude mice, double-replacement clones were indistinguishable from clones expressing only p53B (Figs. 4 and 5, Table 1). Cells obtained by infecting in the other order, that is, p53B-expressing cells infected with Vp53E-Neo, had the same phenotype (*17*). Complete dominance of wild-type p53 activity was observed despite the ~10-fold lower quantities of wild-type than mutated p53 in these cells, an expected consequence of the shorter half-life of wild-type p53.

In sum, single copies of wild-type p53 were sufficient to suppress the neoplastic phenotype of human osteosarcoma cells lacking p53 expression, as well as of cells expressing a mutated p53 allele. These results are consistent with the findings of Baker *et al.* (*20*) that transfection of human wild-type p53 suppressed the growth of human colorectal tumor cells, even those with mutated endogenous p53 alleles. These studies suggest that, like *RB*, human p53 has a broad suppression activity in many tumor types. The dominance of wild-type over mutated p53 in a two-allele configuration suggests that both wild-type p53 alleles must be lost for an oncogenic effect of this gene (*21*). Moreover, transfected wild-type p53 failed to suppress the growth of human colorectal adenoma cells containing wild-type p53 alleles (*20*); similarly, exogenous *RB* failed to suppress osteosarcoma cells with wild-type endogenous *RB* (*3*). Thus the suppressive effect of exogenous *RB* or p53 may be limited to tumor cells lacking wild-type endogenous *RB* or p53. These shared properties of *RB* and p53 reinforce the tumor suppressor gene concept, including the possible clinical use of their replacement in appropriate tumor cells.

One question not answered by previous studies is whether point-mutated p53 has some function, or whether it is completely functionless, that is, is equivalent to its complete deletion. The mutated human p53 allele we used retains some function, because its insertion into osteosarcoma cells augmented their saturation density in culture (*22*). Mutated p53 alleles may confer a growth advantage or a more malignant phenotype to tumor cells without wild-type p53, thereby explaining why mutated p53 alleles are commonly retained in tumor cells. Additional experiments are needed to address this proposal. The idea that mutated p53 has a biological function, and that its function is recessive to that of wild-type p53, is inconsistent with the hypothesis of a dominant negative effect, at least as it applies in natural human tumorigenesis. The dominant transforming properties of mutated murine p53 alleles may be due to the high copy numbers of genes introduced by transfection, and the resulting massive overexpression of mutated p53. Under conditions of equal gene dosage, wild-type p53 is able to override the influence of mutated p53 despite a tenfold molar excess of the latter. These observations may be explained by competition of wild-type and mutated p53 for common cellular targets, for which wild-type p53 is much more avid. In this model, wild-type and mutated p53 would transmit opposite growth signals to these targets, with total absence of p53 perhaps an intermediate signal. Alternatively, mutated p53 may act in an independent pathway to promote selective features of the neoplastic phenotype.

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12. Cloning of wild-type p53 (p53B) cDNA: ~5 μg of fetal brain RNA were mixed with 1.5 μg of oligo(dT) primer and 60 units of avian myeloblastosis virus reverse transcriptase in cDNA buffer (50 mM tris-HCl, pH 8.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM each deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate, and deoxycytidine triphosphate). The reaction mixture was incubated for 90 min at 42°C. After reaction, RNA was degraded with 0.5 M NaOH, and single-stranded cDNA was precipitated with ethanol. PCR amplification was carried out with one-tenth of the cDNA. 100 ng of each oligonucleotide primer (5'-TGCAAGCTTTCCACGACGGTGACACGCT-3' and 5'-AGTGCAGGCCAACTTGTTCAGTGGA-3'), and 5 U of Taq polymerase in PCR buffer (50 mM KCl, 10 mM tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 0.001% gelatin) for 40 cycles in a programmable heat block (Ericomp, San Diego, CA). Each cycle included denaturation at 93°C for 1 min, annealing at 62°C for 80 s, and primer extention at 72°C for 3 min. PCR products were extracted with phenol and precipitated with ethanol. The precipitate was dissolved in H_2O and digested with restriction enzymes (Hind III and Sma I). The p53 cDNA fragment was subcloned into virus vector to form Vp53B-Neo. Subcloned p53B was sequenced by the dideoxy chain termination method [F. Sanger, S. Nicklen, A. R. Coulsen, *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463 (1977)].
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21. One colorectal carcinoma has been described that contains both mutated ($Asp^{281} \rightarrow Gly$) and wild-type p53 alleles (*14*). Possible explanations for this discrepant case are as follows: (i) an intermediate stage of p53 mutation was coincidentally captured, and p53 had not yet contributed to the neoplastic properties of this tumor; (ii) the "wild-type" p53

allele in this tumor actually carried a functionally important mutation outside of the region sequenced (exons 5 to 9); or (iii) some mutated p53 alleles behave differently than others, or differently depending on cell type.

22. That mutated p53 has a function is also suggested by a study [D. W. Wolf, N. Harris, V. Rotter, *Cell* 38, 119 (1984)] in which a presumably mutated p53 gene was transfected into Ab-MuLV–transformed murine leukemia cells that lacked endogenous p53 expression. This resulted in cells with more malignant behavior in tumorigenicity assays.
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27. We thank W. Hammerschmidt and B. Sugden for retroviral vector 477, and E. Harlow for monoclonal antibody pAb421 and p53 cDNA. This study was supported by grants from the National Eye Institute (EY05758 and EY00278) and National Cancer Institute (CA51495), and a fellowship to P.-L.C. from the Ministry of Education, Republic of China.

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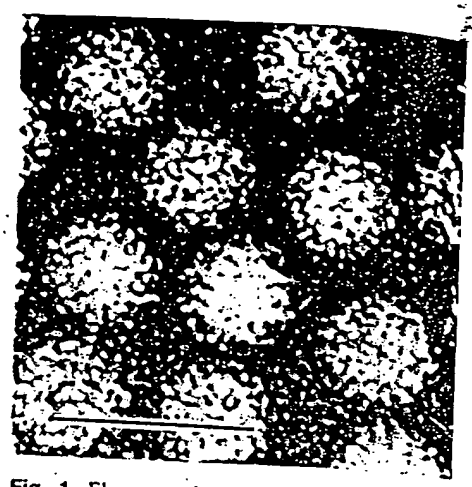

Fig. 1. Electron micrograph of Norwalk virus particles after CsCl gradient purification. Virus was visualized by staining with 1% ammonium molybdate. Scale bar, 50 nm.

Norwalk Virus Genome Cloning and Characterization

XI JIANG, DAVID Y. GRAHAM, KENING WANG,* MARY K. ESTES†

Major epidemic outbreaks of acute gastroenteritis result from infections with Norwalk or Norwalk-like viruses. Virus purified from stool specimens of volunteers experimentally infected with Norwalk virus was used to construct recombinant complementary DNA (cDNA) and derive clones representing most of the viral genome. The specificity of the clones was shown by their hybridization with post- (but not pre-) infection stool samples from volunteers infected with Norwalk virus and with purified Norwalk virus. A correlation was observed between the appearance of hybridization signals in stool samples and clinical symptoms of acute gastroenteritis in volunteers. Hybridization assays between overlapping clones, restriction enzyme analyses, and partial nucleotide sequence information of the clones indicated that Norwalk virus contains a single-stranded RNA genome of positive sense, with a polyadenylated tail at the 3′ end and a size of at least 7.5 kilobases. A consensus amino acid sequence motif typical of viral RNA-dependent RNA polymerases was identified in one of the Norwalk virus clones. The availability of Norwalk-specific cDNA and the new sequence information of the viral genome should permit the development of sensitive diagnostic assays and studies of the molecular biology of the virus.

ACUTE GASTROENTERITIS IS ONE OF the most common illnesses in the United States (*1*, *2*), with a large number (up to 42% of outbreaks) of cases estimated to be caused by Norwalk or Norwalk-like viruses (*3*). Both water- and food-borne transmissions of Norwalk virus have been documented, and particularly large epidemic outbreaks of illness have occurred after consumption of contaminated shellfish including clams, oysters, and cockles (*4*–*10*).

Norwalk virus was discovered in 1973, but knowledge about the virus has remained limited because it has not been possible to propagate the virus in cell culture and suitable animal models have not been found (*2*). Human stool samples obtained from outbreaks and from human volunteer studies have been the only source of virus. Moreover, the concentration of virus in stool is usually so low that virus detection by routine electron microscopy is not possible (*11*–*13*). Current methods of Norwalk virus detection include immune electron microscopy and other immunologic methods such as radioimmunoassays (RIAs) or biotin-avidin enzyme-linked immunosorbent assays (ELISAs), which use sera from humans in acute and convalescent phases. To date, because of either insufficient quantities or unusual properties of the viral antigen, no hyperimmune serum from animals has been successfully prepared. Preliminary biophysical characterization of virions has indicated that particles contain one polypeptide (*14*), but efforts to characterize the viral genome have failed. Therefore, these viruses have remained unclassified and difficult to study. Molecular cloning was seen as an approach to overcome these problems.

To permit better diagnosis and molecular characterization of Norwalk virus, we constructed a cDNA library derived from nucleic acid extracted from virions purified from stool samples obtained from volunteers. Norwalk virus (8FIIa) was administered orally to adult volunteers (*15*, *16*). Stool samples collected after infection were examined for Norwalk virus by either RIAs or direct or immune electron microscopy. Stools from two patients containing the highest amount of Norwalk virus were used as the source of virus for cloning. Norwalk virus was purified by methods used previously for preparation of hepatitis A and rotaviruses from stool, with some modifications (*17*, *18*). Basically, stools containing Norwalk virus were treated with Genetron (1,1,2-trichloro-1,2,2-trifluoroethane) to remove lipid and water-insoluble materials. Virus in the aqueous phase was then centrifuged through a 40% sucrose cushion, and the resultant pellets were suspended, sonicated, and loaded in a CsCl gradient for isopycnic centrifugation. Approximately 10^9 physical particles of virus of relatively high purity (Fig. 1) were obtained from 500 g of stool.

Nucleic acids were extracted from these purified viruses by proteinase K treatment of the samples followed by phenol-chloroform extraction and ethanol precipitation (*19*). Because the nature of the viral genome was unknown, the extracted nucleic acids were denatured with methylmercuric hydroxide before cDNA synthesis. Random primed cDNA was synthesized and a small amount of cDNA was obtained (*19*). Direct cloning of this small amount of cDNA was unsuccessful so a step of amplification of the DNA was performed by synthesizing more copies of the DNA with random primers and the Klenow fragment of DNA polymerase I before cloning. This cloning method was developed based on control experiments in which we amplified a known cDNA fragment of hepatitis A virus (*20*). The proce-

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Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector

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ABSTRACT

A defective adenovirus (Ad) type 5 E1⁻ vector has been combined with the powerful constitutive cytomegalovirus (CMV) major immediate early (IE) promoter to produce a novel eukaryotic expression system. The Ad vector can replicate to high titres in 293 cells and then be used to infect a wide variety of non-permissive cell types. The *Escherichia coli lacZ* and CMV IE1 genes have been cloned to generate the Ad recombinants RAd35 and RAd31 respectively. In human fibroblasts infected with RAd35 β-galactosidase (β-gal) expression could be detected in virtually 100% of target cells, there was no detectable transcription from the Ad genome and extremely high levels of expression could be achieved with β-gal representing the predominant cytoplasmic cellular protein. Additionally, a number of agents, including the CMV IE1 gene product (in RAd31) and forskolin, significantly enhanced expression from RAd35-infected human fibroblasts. Lower levels of constitutive β-gal expression were obtained in RAd35-infected HeLa cells but again expression could be enhanced (up to 60 fold) by chemical inducing agents. Expression from the IE promoter in the Ad vector could be repressed by co-infection with CMV.

INTRODUCTION

High level expression of recombinant proteins can readily be achieved using Adenovirus (Ad) vectors when expression is being driven by the Ad major late promoter (reviewed in 4). Ad vectors have been produced based on E1 deletion mutants. Since the E1 gene trans-activates early phase gene expression, Ad E1⁻ mutants can replicate in helper cell lines carrying integrated E1 helper function but in other cell types early phase gene expression is not activated. This paper describes the novel combination of the powerful constitutive CMV major immediate early (IE) promoter with a defective Ad vector. The Ad vector acts as an extremely efficient gene delivery system to non-permissive target cell populations where high level expression of genes cloned under the control of the CMV IE promoter can be achieved.

The strength of the CMV major IE promoter lies primarily in its enhancer element which contains a remarkable array of 16, 18, 19 and 21 bp imperfect direct repeats (1,5). The 18 and 19 bp repeats bind transcription factors with the properties of NF-κB and the cAMP responsive binding protein (CREB) respectively (10,27,30). Induction of NF-κB-binding activity in Jurkat cells by phorbol ester treatment stimulates the IE promoter (27), while agents which increase CREB activity have been shown to enhance expression via the 19 bp repeat (6,15,22,30). Additionally the IE promoter can be stimulated in trans by the products of the CMV major IE gene itself (8) and the Ad E1a gene (9,11,22). We have exploited the inducible properties of the CMV promoter in the Ad vector to significantly enhance yields of an expressed protein.

MATERIALS AND METHODS

Cells

Primary human lung fibroblasts (MRC5 cells) and 293 cells (13) were grown in Glasgow's modified minimal essential media (Imperial Laboratories, Andover, UK) containing 8% foetal calf serum. All Ad stocks were titrated in 293 cells. MRC5 cells were stimulated by incubating in the presence of 50 ng/ml phorbol-12-myristate-13-acetate (PMA) and 4 μg/ml phytohemagglutinin (PHA). 10 μM forskolin, 1mM dibutyryl cAMP or 2mM sodium butyrate.

Construction of RAd35 and RAd31

Ad recombinants were produced according to the methodology described by McGrory and co-workers (19). The *E. coli lacZ* gene was inserted into a transient expression vector under the control of the CMV major IE promoter (−299 to +69) and upstream of a polyadenylation signal (+2757 to +3025). Nucleotide sequence numbering of the CMV strain Ad169 major IE gene is as previously described (1). The CMV IE promoter/*lacZ* expression cassette was excised from the transient expression vector on a *Hin*dIII fragment and inserted into the Ad transfer vector pMV60 to generate the plasmid pMV35 (Fig 1a). pMV60 is identical to pXCX2 (29) except that a linker (containing the *Hin*dIII cloning site) has been inserted at its unique *Xba*I cleavage site.

pMV35 and pJM17 were co-transfected into 293 cells and after 7 days Ad plaques were detected. Plasmid pJM17 contains the entire Ad5dl309 genome with the prokaryotic vector pBRX inserted into the E1a gene. The prokaryotic vector insertion makes pJM17 too large to package into Ad nucleocapsids (19).

Following recombination the IEP/*lacZ* expression cassette replaced both the prokaryotic vector and the Ad E1a gene region thus generating a smaller DNA molecule which could replicate and be packaged in the helper cell line (Fig 1a). A preliminary analysis of the Ad recombinant RAd35 has shown it to express β-gal in both MRC5 and 293 cells (33).

A second Ad recombinant containing the CMV major immediate early gene (IE1) under the control of its own promoter was constructed using the same methodology. A cDNA copy of the IE1 gene (1) was used and the IEP/IE1 expression cassette was inserted into pMV60 to produce pMV31. IEP/*lacZ* and IEP/IE1 were cloned into the Ad transfer vector in opposite orientations. Consequently, in RAd31, transcription of the IE1 gene is being driven in a right to left direction with respect to the conventional orientation of the Ad genome (Fig 1b).

β-Galactosidase assay

The expression of β-galactosidase (β-gal) was detected either by direct histological staining of cells with the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (35) or by performing a quantitative enzyme assay on cell extracts using ONPG (*o*-nitrophenyl- β-D-galactopyranoside) according to a standard protocol (26) except the reaction volume was scaled down to 100μl. The assay was performed in a microtitre plate and absorbence measured using a Multiskan MCC plate reader at 414 nm.

Immunofluorescence

MRC5 cells grown on glass coverslips were infected with RAd31 (10 PFU/cell). Acetone-fixed cells were incubated with the anti-IE1 monoclonal antibody L14.94 (kindly provided by Dr Jay Nelson) for 1h at 37°C, washed in PBS then incubated with FITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:50 in PBS for 30 min at 37°C. Coverslips were washed in PBS before mounting.

Northern transfer

Total cytoplasmic RNA was purified and Northern transfer experiments were performed as described previously (34).

RESULTS

Construction of Ad recombinants and constitutive expression

In the construction of the recombinant viruses RAd35 and RAd31 the Ad E1 gene region was replaced by the expression cassettes IEP/*lacZ* and IEP/IE1 as detailed in Fig 1. Consequently, both Ad recombinants are E1 deletion mutants which require the provision of a helper function for replication. The recombinant viruses were plaque purified and high titre virus stocks prepared in 293 cells. Restriction endonuclease cleavage of DNA purified from both recombinant viruses confirmed the insertion of appropriately-sized fragments (results not shown).

The host range of CMV is extremely limited with an efficient productive infection in vitro being observed only in primary human fibroblasts. In contrast, the CMV IE promoter itself can function in a wide variety of cell types (17,21,28). Regulation of expression from the IE promoter in the Ad recombinants was investigated both in cell lines permissive (MRC5 cells) and non-permissive (HeLa cells) for CMV. HeLa cells are unusual in that following CMV infection there is no detectable IE gene expression. Nevertheless, the CMV IE promoter will function in HeLa cells when introduced by DNA transfection or in a

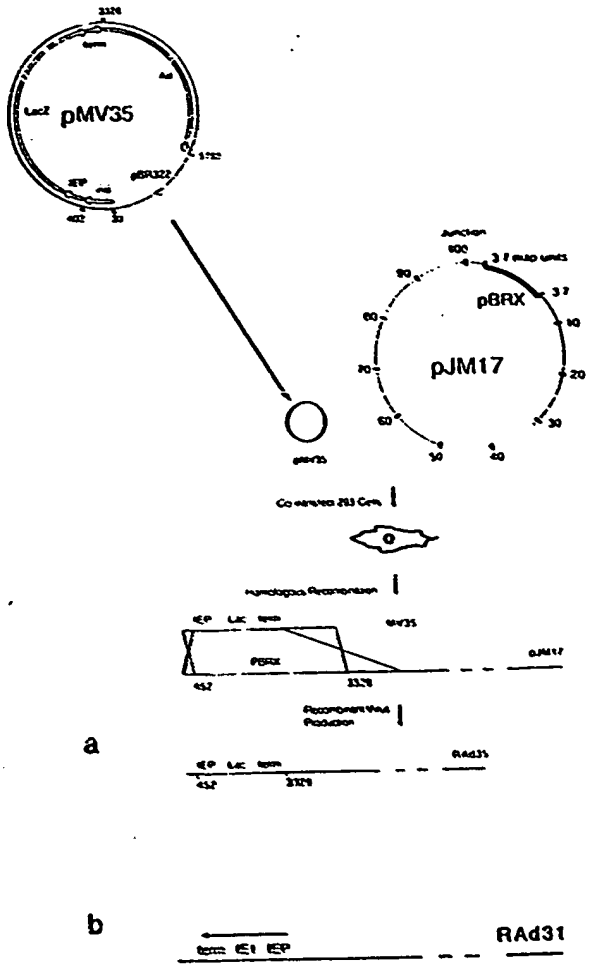


Figure 1. Generation of recombinant viruses RAd35 and RAd31. (a) The CMV IE promoter/*lacZ* expression cassette was inserted into an Ad transfer vector to generate the plasmid pMV35. The transfer vector provides the expression cassette with flanking homology from either side of the Ad E1a gene region. Numbers indicated on pMV35 refer to the Ad5 genomic sequence. pJM17 contains the entire Ad5dl309 genome. Plasmids pJM17 and pMV35 were co-transfected into 293 cells. Homologous recombination between the plasmids resulted in the IEP/*lacZ* cassette replacing both the E1a gene region (bases 402–3328) and the intervening prokaryotic vector. The resulting E1a$^-$ recombinant was designated RAd35. (b) The same protocol was used to generate RAd31. The CMV/IE1 cassette was inserted into the Ad transfer vector producing the plasmid pMV31 which was co-transfected into 293 cells with pJM17. The expression cassettes were inserted into RAd35 and RAd31 in opposite orientations.

replicating Ad vector (14). MRC5 and HeLa cells were infected with RAd35 and expression of the *lacZ* reporter gene assayed by directly staining cells with the chromogenic substrate X-gal. When MRC5 cells were infected at high multiplicity of infection (MOI) histological staining demonstrated β-gal expression in virtually 100% of the target cell population (Fig 2a&b). The Ad vector clearly functions as a highly efficient gene delivery system in primary human fibroblasts. However, expression of β-gal in

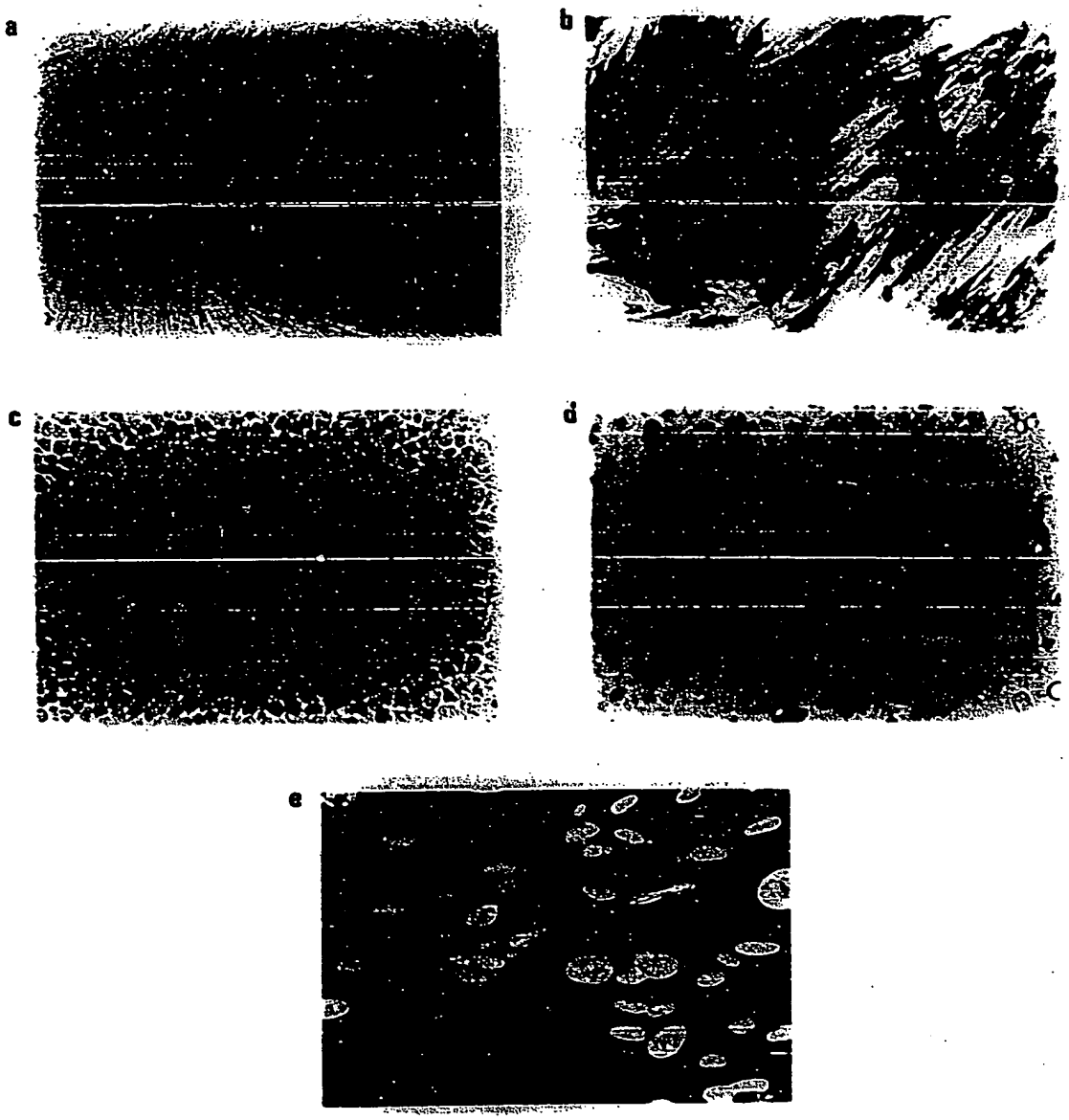


Figure 2. Expression from RAd35 and RAd31 in non-permissive cells. (a) Uninfected MRC5. (b) RAd35-infected (30 PFU/cell) MRC5 cells. (c) uninfected HeLa cells and (d) RAd35-infected (30 PFU/cell) HeLa cells were stained 48h p.i. for β-gal activity using the chromogenic substrate X-gal. The RAd35-infected cells stained a dark blue colour. (e) Nuclear expression of the CMV IE1 gene product in RAd31-infected MRC5 cells (10 PFU/cell) detected 16h p.i. by indirect immunofluorescence.

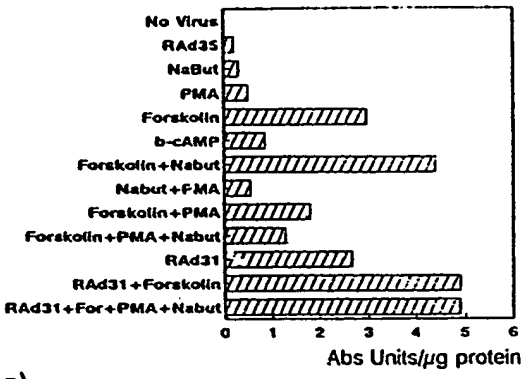
RAd35 infected HeLa cells was relatively low. Direct staining of monolayers with X-gal revealed that even at high MOI only approximately 10% of cells expressed β-gal at a detectable level (Fig 2 c&d).

Although prepared by the same methodology used to generate RAd35, the IEP/IE1 expression cassette was inserted into RAd31 in the opposite orientation (Fig 1b). To test for constitutive expression, RAd31-infected MRC5 cells were stained with an anti-IE1-specific monoclonal antibody. The characteristic nuclear immunofluorescence pattern typical of the CMV major IE protein was observed in almost all cells (Fig 2e). In a parallel control experiment performed with uninfected and RAd35-infected MRC5 cells, no significant immunofluorescence could be detected (results not shown). Infection of MRC5 cells with RAd31 was observed to induce a cytopathic effect (CPE) even at low MOI. The CPE was distinct from that induced by a wild-type Ad

infection and the results of RNA hybridisation experiments (described below) indicated the CMV IE1 gene product was unable to complement the Ad E1 deletion. This result is in agreement with published data from DNA transfection experiments (32).

Enhanced expression

Expression from the CMV IE promoter can be stimulated by a number of agents; most notably the IE1 gene product and by inducers of the transcription factors NF-κB (e.g. PMA/PHA) and CREB (e.g. forskolin and dibutyryl cAMP) which are believed to bind to the enhancer. The effect of such inducing agents on the CMV IE promoter in the Ad vector was investigated. Substantial enhancement of expression was achieved in chemically stimulated RAd35-infected MRC5 and HeLa cells (Fig 3). The most pronounced effect by a single agent was produced by forskolin in MRC5 cells where a 14-fold stimulation in β-gal expression levels was observed. Dibutyryl-cAMP routinely stimulated RAd35 expression less efficiently than forskolin. In MRC5 cells PMA/PHA treatment produced only a slight stimulation in expression and, indeed, had a negative effect when used in combination with forskolin. The pattern of stimulation by chemical agents in HeLa cells was quite different. None of the agents alone increased expression dramatically, PMA/PHA treatment producing the most significant increase (4-fold). There was, however, a synergistic effect when any two agents were used in combination. When sodium butyrate, forskolin and PMA/PHA were added together expression was stimulated 60-fold (Fig 3b). Sodium butyrate is an inhibitor of DNA replication which is known to modify chromatin structure. It has been shown to both stimulate expression from the SV40 and RSV promoters in DNA transfection experiments (12) and to induce CMV infection of epithelial cells (24,31). In both MRC5 and HeLa cells sodium butyrate had a positive effect on expression levels, especially when used in combination with other inducing agents.



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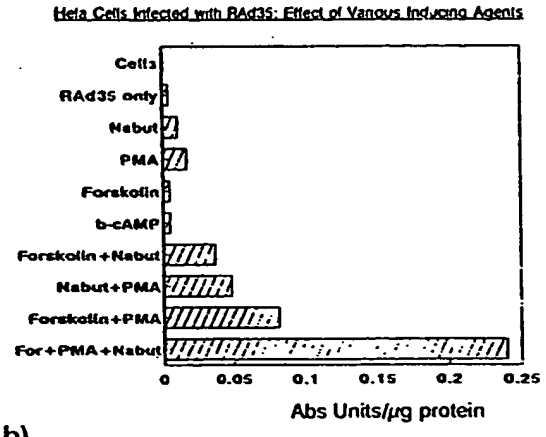

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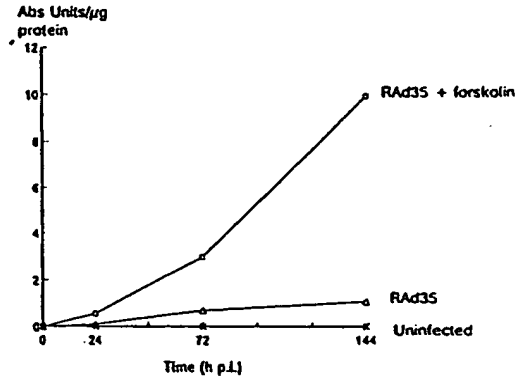
Figure 3. Enhanced expression in RAd35-infected cells. a) MRC5 cells and b) HeLa cells infected with RAd35 (30 PFU/cell) and treated with inducing agents: 2 mM sodium butyrate (NaBut), 50ng/ml PMA was used in combination with 4 μg/ml PHA (PMA), 10 μM forskolin and 1 mM dibutyryl c-AMP (b-cAMP). Inducing agents were added immediately after Ad infection. In co-infection experiments RAd31 was also used at 30 PFU/cell. ONPG assays were performed on cellular extracts taken 48h p.i.



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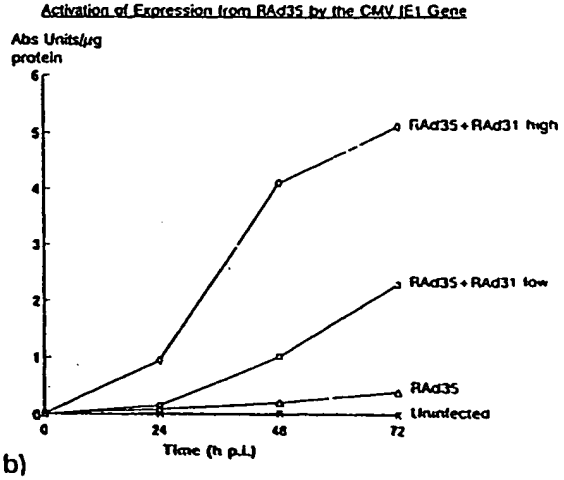

b)

Figure 4. Temporal control of expression from RAd35. RAd35 infected (30 PFU/cell) MRC5 cells were treated with (a) 10 μM forskolin or (b) co-infected with RAd31 at low (3 PFU/cell) and high (30 PFU/cell) MOI and samples taken at the times indicated. Samples were also taken for mock-infected and RAd35-infected cells in both experiments. β-Gal enzyme activity in cell extracts was measured using an ONPG assay.

In MRC5 cells, RAd31 co-infection stimulated expression from RAd35 12-fold indicating that the expressed IE1 gene product was biologically active in the context of a virus infection. The IE1 gene product (expressed by RAd31) can stimulate the IE promoter in human fibroblasts, possibly via the 18bp repeat (8), while forskolin presumably stimulates the interaction of CREB with the 19 bp repeat (6,15,30). In MRC5 cells, the highest level of β-gal expression from RAd35 was observed when RAd31 and forskolin were used in combination (23-fold stimulation).

Temporal regulation of expression

During a productive CMV infection the IE promoter is active only for the first 6 h after which transcription is repressed (20), possibly by the action of the CMV IE2 gene product. A series of time course experiments were performed to investigate temporal regulation of expression from the IE promoter in the Ad vector. The level of β-gal expressed by RAd35 in MRC5 cells continues to increase until at least 144h p.i. (Fig 4&5). In transient DNA/calcium phosphate transfection experiments maximum expression levels are reached at approximately 48 h post transfection. The Ad vector, however, appears to hold the IEP/*lacZ* expression cassette in a transcriptionally active state much longer. Furthermore forskolin-enhanced expression continued throughout the duration of the time course experiment (Fig 4a). Co-infection of RAd31 with RAd35 also enhanced expression of β-gal from the IE promoter up to 72h p.i., with the stimulatory effect of the IE1 gene product being dosage dependent (Fig 4b). After the 72h time point RAd31 co-infection began to induce cell death.

CMV encodes factors which can both stimulate and repress expression from the IE promoter. CMV co-infection effectively repressed β-gal expression from RAd35 (Fig 5). Since CMV infection can complement an Ad E1a defect (32), cytosine β-D-arabinofuranoside (ara-C) was used in this experiment to inhibit Ad replication. The repressive effect of CMV co-infection on RAd35 expression was even more pronounced when Ad replication was being inhibited. By contrast, treatment with ara-C by itself stimulated expression from RAd35-infected cells 2-fold in the absence of CMV co-infection (result not shown). The IE promoter used in the Ad vector extends from −299 to +69 and thus contains the cis-acting element identified as the site of CMV IE2-induced repression (7,18,23). The IE promoter in the RAd35 genome was being negatively regulated during the early and late phases of infection co-ordinately with the IE promoter in the CMV genome.

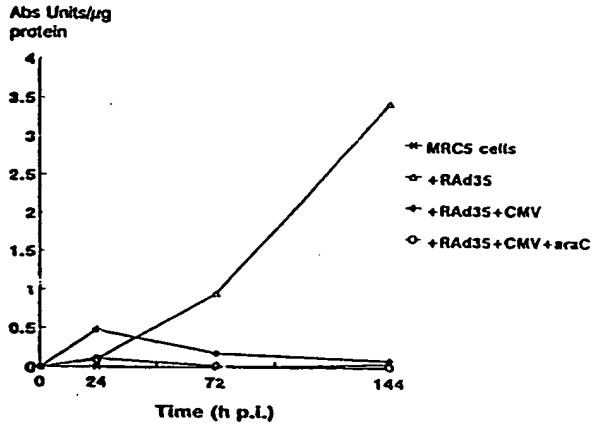

Figure 5. Effect of CMV co-infection on expression from RAd35. In a time course experiment the effect of CMV infection (5 PFU/cell), with and without Ara-C (20 μg/ml), on β-gal expression in RAd35-infected (30 PFU/cell) MRC5 cells was investigated. β-Gal enzyme activity in cell extracts were measured using an ONPG assay.

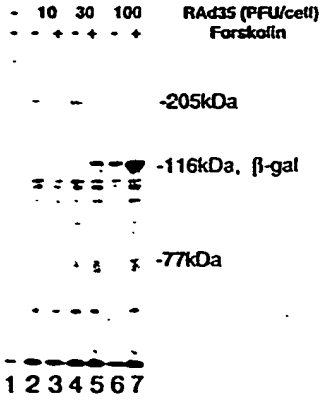

Figure 6. Visualisation of expressed protein on a polyacrylamide gel. MRC5 cells in 6 cm diameter dishes were infected with RAd35 at 10, 30 and 100 PFU/cell with and without forskolin (10μM) induction and incubated 144h. Total cytoplasmic cell extracts were prepared and samples subjected to SDS-PAGE (7.5% gel). Protein gels were stained using Coomassie blue.

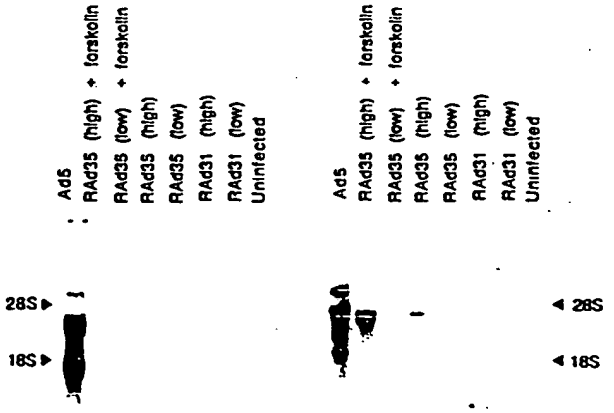

Figure 7. RNA hybridisation experiment to test for Ad transcription in nonpermissive human fibroblasts. Total cytoplasmic RNA was extracted from uninfected cells and MRC5 cells infected with: non-defective Ad5dl309 (1 PFU/cell), RAd35 at low (10 PFU/cell) and high (100 PFU/cell) MOIs, with and without forskolin induction, and RAd31 at low (3 PFU/cell) and high (30 PFU/cell) MOIs. RNA was prepared 72h p.i. The RNA was then subjected to electrophoresis in 1% formaldehyde agarose gels, blotted on to nitrocellulose membranes and hybridised with radiolabelled DNA probes. The resulting autoradiographs are shown: a) hybridised with a pIM17 probe and b) hybridised with a pIM17 probe then rehybridised with a probe containing the *lacZ* gene (pON3).

Expression Levels

Expression from the CMV IE promoter in the Ad vector has been shown to increase appreciably with time and to be strongly stimulated by certain inducing agents. We were interested in determining what levels of expression could be achieved with this vector system. MRC5 cells were therefore infected at 10, 30 and 100 PFU/cell with RAd35 then incubated for 144h both with and without forskolin induction. Cytoplasmic protein extracts were prepared and analysed by SDS-PAGE. Expression from RAd35 was proportional to the size of the input virus inoculum with or without forskolin induction (Fig 6). The level of expression detected in non-permissive cells from the constitutive CMV IE promoter was extremely high for a non-replicative system. An induced protein corresponding to β-gal can be clearly identified in Fig 6 lanes 5&6 and in lane 7 it is the predominant protein species. A densitometric analysis of lane 7 indicated that β-gal represented 17.5% of total cytoplasmic cellular protein giving an estimated yield of approximately 27μg β-gal for a 6 cm diameter tissue culture dish in this sample. In similar experiments β-gal expression levels of greater than 35% total cytoplasmic cell protein have been achieved (not shown).

Restriction of Ad Vector Expression

The primary purpose in developing the vector system was to use Ad as an efficient gene delivery system to produce expression only of the gene cloned under the control of the CMV IE promoter. It was important, therefore, to investigate whether there was significant breakthrough to Ad early and late phase gene expression in the non-permissive cell line. Total cytoplasmic RNA was prepared from MRC5 cells infected with RAd35, RAd31 and a non-defective Ad5. In a hybridisation experiment, a radiolabelled pJM17 probe (containing the complete Ad genome) was able to detect Ad-encoded RNAs in MRC5 cells infected with the non-defective Ad but not in cells infected with RAd35 (with or without forskolin stimulation) or RAd31 (Fig 7a). Ethidium bromide staining demonstrated similar amounts of RNA were present in each track (not shown). To further test that mRNAs purified from RAd35-infected cells were intact a hybridisation probe containing the *lacZ* gene was also used (Fig 7b). A major RNA species of approximately 3.3kb was detected in RAd35-infected cells whose abundance was proportional to the input MOI and enhanced by forskolin. In MRC5 cells infected with both RAd35 and RAd31 there was no evidence of breakthrough into Ad gene expression. This experiment also confirmed that the CMV IE1 gene in RAd31 did not complement the E1 deletion in the Ad vector.

DISCUSSION

The CMV IE promoter was combined with a defective Ad vector to express recombinant gene products in the target cell population without: (i) having to accommodate the artificial conditions and inefficiency associated with DNA transfection, (ii) the need to clonally select continuous cell lines or (iii) interference from vector gene functions. Ad type 5 is a particularly appropriate vector to use as a gene delivery system. High titre Ad5 stocks ($>10^9$ PFU/ml) can readily be produced, an efficient technology has been developed to generate recombinants (19) and the virus can infect an exceptionally wide range of cell types. RAd35 has been shown to infect and induce β-gal expression in human lymphocytic lines (JM, C8166 and U937 cells), primary mouse macrophages, primary chick embryo fibroblasts, 293, Vero, porcine kidney and Chinese hamster ovary cells (unpublished results).

Expression from RAd35, detectable in virtually 100% of infected MRC5 cells, was proportional to the size of the virus inoculum and continued to increase with time up until at least 144h p.i. The efficiency of expression was much less efficient in HeLa cells. Since they are highly susceptible to Ad infection, HeLa cells presumably either lack factors necessary for activating or contain factors capable of repressing the IE promoter. Levels of expression attainable in different cell types will be dependent on the susceptibility of cells to Ad infection combined with either the constitutive or the inducible activity of the CMV IE promoter.

We were interested in determining whether the expression system could be used to study the control of gene expression. The ability of (i) the CMV IE1 gene product encoded by RAd31 to trans-activate expression from RAd35 and (ii) CMV co-infection to repress expression was clearly demonstrated. The cytotoxicity associated with RAd31 infection suggests the IE1 gene product may have a role in generating the CPE associated with the early phase of CMV infection (2). In addition to being a transcriptional trans-activator, the CMV IE1 gene also encodes a primary target for cytotoxic T lymphocytes (3). The recombinant RAd31 will facilitate further studies into the biological properties of the CMV major IE protein.

The CMV IE promoter in the context of the Ad vector was susceptible to stimulation by a variety of chemical agents. Interestingly, the effect of such inducing agents on the promoter in MRC5 and HeLa cells was quite different. Forskolin clearly significantly enhanced expression from the Ad-based expression system in MRC5 cells (although not in HeLa cells) and potentially could be used to increase the yield of any recombinant protein. Results obtained with RAd35 are not in complete agreement with data from DNA transfection experiments. Most notably, Stamminger and co-workers (30) observed that cAMP stimulation increased expression 5.2-fold in HeLa cells but no enhancement was detected in human fibroblasts. If such differences are due to the method of gene delivery, then the results obtained with RAd35 are important to studies of CMV pathogenesis. However, the Ad vector contains a relatively weak enhancer element, normally associated with the Ad E1 gene, located upstream of the CMV enhancer. This additional enhancer element may influence expression from the CMV promoter.

The combination of the CMV IE promoter with a defective Ad vector has definite advantages over comparable expression systems and can clearly be applied to studies of gene regulation, gene function and antigen presentation. A similar Ad recombinant has recently been used to generate an antibody response in vaccinated mice and provide protection against a viral challenge (16). This novel, efficient expression system has great potential for use in vaccine development and possibly also for use in somatic gene therapy (25). The defective Ad vector inflicts minimal damage on the target cell population, compared with lytic vaccinia systems, and provides a degree of biological containment. The exceptionally high levels of β-gal detected in human fibroblasts infected with RAd35 exceeded expectation and makes the system attractive simply to achieve high level production of recombinant proteins.

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Construction of a recombinant adenovirus containing the *denV* gene from bacteriophage T4 which can partially restore the DNA repair deficiency in xeroderma pigmentosum fibroblasts

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The *denV* gene from bacteriophage T4 encodes a pyrimidine dimer-specific endonuclease that has the capacity to initiate excision repair of DNA. Cells from excision repair-deficient xeroderma pigmentosum (XP) patients are able to carry out excision repair initiated by the *denV* gene product and introduction of the *denV* gene into XP cells results in the partial restoration of colony-forming ability after irradiation with UV light. In this work we have constructed a helper-independent recombinant human adenovirus, Ad5denV, which contains the *denV* gene. A 1.9 kb cartridge consisting of the *denV* gene flanked by the long terminal repeat (LTR) promoter from Rous sarcoma virus (RSV) and the simian virus 40 (SV40) polyadenylation (poly A) splice signals, was inserted into the E3 region of an E3 deletion mutant (Ad5d1E3) of adenovirus type 5. Infection of human fibroblasts and other permissive human cells with Ad5denV resulted in lytic infection and expression of the *denV* gene was confirmed by primer extension of infected cell RNA. The ability of the *denV* gene to restore the DNA repair deficiency in XP fibroblasts was examined using host cell reactivation of viral structural antigen formation for UV-irradiated adenovirus. The control virus, Ad5VSV, was also a recombinant which contained the gene for vesicular stomatitis virus glycoprotein G inserted into the E3 region of Ad5d1E3. UV survival of Ad5denV was similar to that of Ad5VSV following infection of two normal fibroblast strains and a Cockayne syndrome fibroblast strain, CS7SE, from complementation group B. In contrast, UV survival of Ad5denV was significantly greater than that for Ad5VSV after infection of three unrelated XP fibroblast strains from complementation groups A, C and E. However, UV survival of Ad5denV in the XP fibroblasts did not reach levels obtained in normal fibroblasts, indicating that restoration of the XP defect was partial.

Introduction

The cloning and characterization of several prokaryotic and eukaryotic genes involved with DNA repair is an exciting development of the last decade, and is expected to help considerably in our understanding of the DNA repair deficiencies associated with a number of human diseases (1).

Xeroderma pigmentosum (XP*) is an autosomal recessive disease in humans characterized by an extreme sensitivity to UV light and a predisposition to sunlight-associated skin cancer (2). Cultured fibroblasts from XP patients are hyposensitive to the lethal, mutagenic and transforming effects of UV light (3–6). The majority of available XP fibroblast strains have been assigned to seven or eight mutually complementing groups, designated A–H (2–4,7–9), and have some defect in the incision step of nucleotide excision repair that acts to remove bulky DNA lesions. The excision repair defects of XP cells have been reported for both the cyclobutane pyrimidine dimer and the [6–4] pyrimidine–pyrimidone photoproducts (10–14). The complexity of the initial incision step may be inferred from the identification of at least seven distinct XP complementation groups that are defective in the incision step. In contrast, the *denV* gene product from bacteriophage T4, a pyrimidine dimer–DNA glycosylase with an associated apyrimidinic endonuclease activity, has the capacity to initiate DNA repair as a single enzyme by incising specifically at pyrimidine dimers (15,16). *In vitro* as well as *in vivo* studies have shown that XP cells are able to carry out excision repair of dimers initiated by endonuclease V (17–20). The *denV* gene of bacteriophage T4 has been cloned and the DNA sequence determined (21,22). Introduction of the *denV* gene into a simian virus 40 (SV40)-transformed XP fibroblast line (23) as well as an XP–HeLa hybrid cell line (24) was found to restore partially resistance of the XP cells to UV irradiation.

One major obstacle in experiments aiming at stable complementation of DNA repair-deficient human cells is the scarcity of immortalized DNA repair-deficient human cells. Furthermore, it is apparent that several tumor and SV40-transformed human cells show a deficiency in a number of DNA repair pathways (25–29). This suggests that the use of SV40-transformed human XP cells and XP–HeLa hybrid cells as recipients of DNA repair genes in DNA-transfection experiments may preclude the restoration of 'normal' levels of DNA repair.

Host cell reactivation (HCR) of adenovirus (Ad) treated with physical or chemical agents is a sensitive and quantitative measure of the DNA repair capacity of human cells and has been successfully applied in the detection of DNA repair defects for a number of different XP fibroblast strains (30–32). Adenovirus has broad host range, making it a suitable vector for the study of mammalian gene expression, and a number of recombinant adenovirus vectors have been constructed that express various inserted foreign genes (33–39).

Combining the use of adenovirus as a mammalian expression vector and UV survival of adenovirus as a measure of the DNA repair capacity of the infected cell would allow an examination of the ability of a DNA repair gene to complement the repair deficiency of untransformed primary cell lines.

In order to examine the potential use of adenovirus for studying the expression of cloned DNA repair genes in mammalian cells we have constructed a recombinant adenovirus containing the

*Abbreviations: XP, xeroderma pigmentosum; SV40, simian virus 40; HCR, host cell reactivation; Ad, adenovirus; MEM, minimal essential medium; VSV, vesicular stomatitis virus; RSV, Rous sarcoma virus; LTR, long terminal repeat; Vag, viral structural antigens; CS, Cockayne syndrome.

denV gene and examined the UV survival of this virus in several different human fibroblast strains including excision repair-deficient fibroblast strains from patients with XP. Our results show a partial restoration in the survival of UV-irradiated adenovirus due to expression of the *denV* gene following infection of XP fibroblasts from complementation groups A, C and E.

Materials and methods

Cells

Stock monolayers of diploid human fibroblasts as well as human 293 cells were grown in screw-cap bottles (Falcon Plastic) and placed in a CO_2 incubator at 37°C and 90–100% humidity. The growth medium was Eagles's alpha-minimal essential medium (alpha-MEM) supplemented with 10% fetal bovine serum together with antibiotics. The normal fibroblast strain GM2803, as well as the Cockayne syndrome fibroblast strain CS7SE from complementation group B and the XP fibroblast strains XP12BE (GM5509, group A), XP2BE (GM677, group C) and XP2RO (GM2415, group E) were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ, USA. The normal strain B. Hanes (B.H.) was obtained from Dr Pat Chang, Department of Pediatrics, McMaster University, Hamilton, Ontario, Canada. Fibroblast cell cultures were generally confluent by 7–9 days following a split ratio of 1:3.

293 cells are a transformed human cell line originally obtained by transfection of human embryonic kidney cells with sheared human adenovirus type 5 DNA (40) and were obtained from Dr Frank L.Graham, Departments of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada.

Virus

Viral stocks on Ad5dl309 (41), Ad5VSV (38) and Ad5denV were grown and titrated on human 293 cells as described previously (35,40). Ad5dl309 is the parental virus used in construction of both Ad5VSV and Ad5denV. Ad5VSV was constructed from Ad5dl309 by removal of part of the E3 region of the virus between the *Xba*I sites at positions 78.5% and 84.7% on the Ad5 genome, and subsequent insertion of a 2.1 kb fragment containing the glycoprotein G from vesicular stomatitis virus (VSV) (38). The initial stocks of Ad5dl309 and AD5VSV were generously supplied by Dr L.Prevec, Departments of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada.

Plasmids

pFGDX1 contains the right end sequences of Ad5dl309 from the *Bam*HI site at 59.5 to 100%, except for the Ad5 *Xba*I D fragment from 78.5 to 84.3% (35).

pRSVdenV (23) contains the Rous sarcoma virus (RSV) long terminal repeat (LTR) of pRSVcat (42) on a 578 bp *Nde*I–*Hin*dIII fragment followed by 6 bp (AGCTTG) from the *Hin*dIII–*Sph*I linker of pEMBL19 (43), the 475 bp *Cla*I fragment of pdenV-52 (43), and 11 bp (CGACTCTAGAG) from the *Acc*I–*Bam*HI linker of pSP65 (Promega Biotec, Madison, WI), cloned into the ~4.5 kb *Bgl*II–*Nde*I fragment from pSV2dhfr (45), containing the SV40 large T splice and polyadenylic acid signals, as well as portions of pBR322. The *denV* gene and the junctions of pRSVdenV have been previously sequenced and confirmed to be intact (23).

pEMBL18/pRSVdenV was constructed by inserting the 1.9 kb *Nde*I–*Bgl*II fragment of pRSVdenV into the *Xba*I site of pEMBL18. Firstly, the *Xba*I site contained in the *denV* sequence of pRSVdenV was deleted with Klenow and the resultant plasmid was digested with *Bgl*II and *Nde*I. The *Bgl*II–*Nde*I 1.9 kb fragment, containing the RSV and *denV* sequences plus the SV40 large T splice and polyadenylation signals was isolated, blunt ended with Klenow, *Xba*I linkers added to both ends and inserted into the *Xba*I site of pEMBL18 (43).

Purification of plasmid DNA was by buoyant density centrifugation in CsCl–ethidium bromide gradients.

Rescue of the denV gene in adenovirus type 5

DNA from Ad5dl309 was extracted from gradient-purified virus as described elsewhere (34). After complete digestion with *Eco*RI, 5–10 μg of this DNA was transfected into 293 cells using the calcium phosphate precipitation method of Graham and van der Eb (46) along with 5–10 μg of plasmid pMCADV linearized with *Sal*I (Figure 1). The resultant plaques were isolated and expanded into ministocks and viral DNA was isolated for restriction enzyme analysis as described previously (35). One recombinant with the expected *Hin*dIII restriction pattern and designated Ad5denV was selected for further study. The virus was plaque purified by two successive plaque titrations on 293 cells and viral stocks grown from 293 cells.

Detection of denV DNA in Ad5denV

Identification of DNA sequences specific to the *denV* cartridge (Figure 2) was carried out using restriction fragment analysis of DNA on 0.8% agarose gels together with Southern blotting to nitrocellulose (47) and subsequent hybridization with DNA from the *denV* cartridge, which had been labelled *in vitro* with [α-^{32}P]dCTP by nick translation. Radioactive labelling of gel-extracted DNA fragments was carried out using a standard nick translation kit from Bethesda Research Laboratories, and unincorporated nucleotides were removed by passage through a G-50 spun column (48).

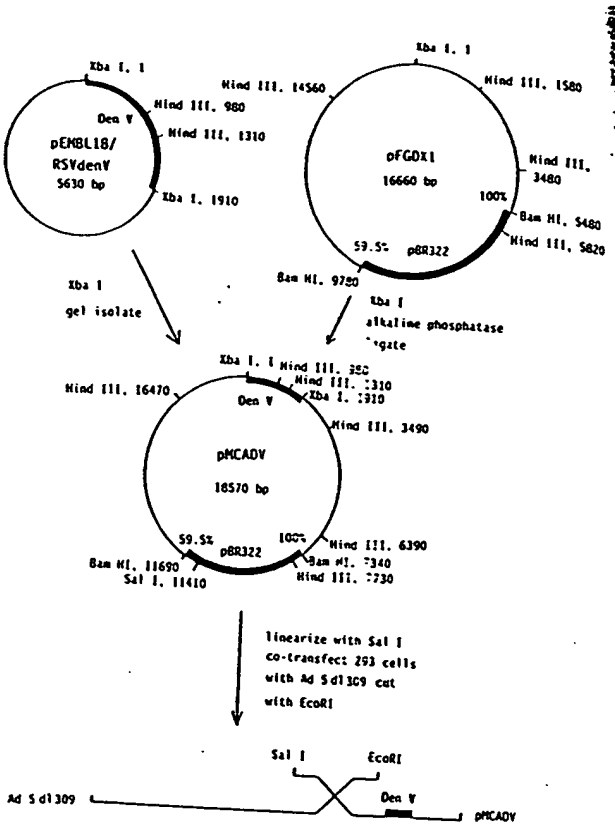

Fig. 1. Construction of Ad5denV. Plasmid pEMBL18/RSVdenV was digested with *Xba*I and the 1.9 kb fragment containing the *denV* expression cartridge was isolated by extraction from a polyacrylamide gel. The fragment was then ligated to plasmid pFGDX1 which had been digested with *Xba*I and treated with alkaline phosphatase. The ligation mixture was used to transform *E.coli* strain HB101, and ampicillin-resistant colonies were screened for plasmids with inserts. The resulting plasmid, pMCADV, was linearized with *Sal*I, mixed in varying ratios with Ad5dl309 that had been digested with *Eco*RI, and used to transfect human 293 cells. Of 24 viral plaques arising from the transfection, three were recombinants, one of which contained the required *denV* insert and was designated Ad5denV. Solid areas on plasmids represent the *denV* cartridge and pBR322 sequences.

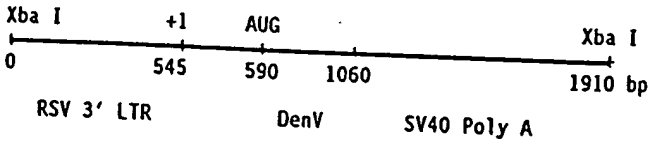

Fig. 2. The *denV* expression cartridge. The cartridge consisted of the following elements: a 55 bp fragment from pBR322 followed by 524 bp from the 3' LTR of RSV. The TATA box of the RSV promoter is at position 510 in the cartridge sequence and the reported start of transcription is at position 545. The *denV* coding sequence begins at the start codon at position 591, and the TAA opal stop is found at position 1060. After translation termination, there is part of the SV40 small t antigen intron followed by the large T antigen polyadenylation splice site derived from the pSV2 plasmid series.

Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase and DNA polymerases were purchased from Bethesda Research Laboratories or Boehringer-Mannheim Biochemicals and used as directed by the manufacturer. All chemicals were from Sigma (St Louis, MO) unless otherwise noted.

Primer extension

Oligonucleotides were synthesized by the Central Facility of the Institute of Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada. The oligonucleotide used for the present study, was a 24mer

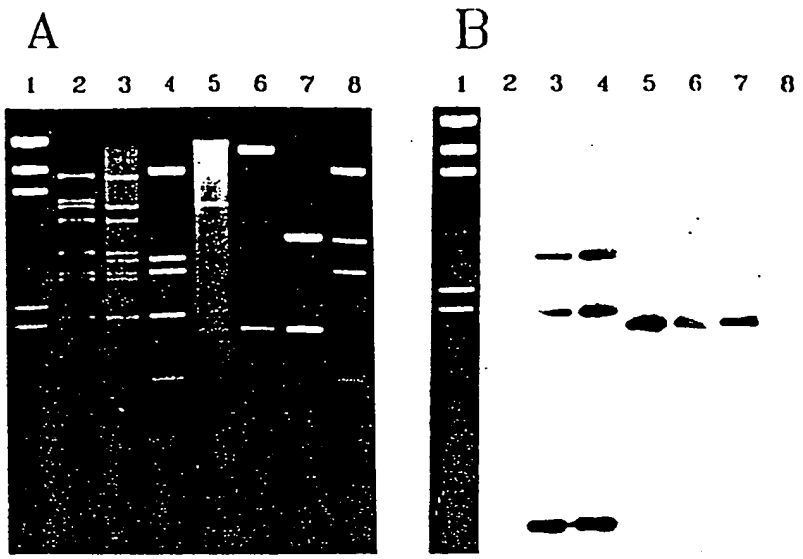

Fig. 3. Restriction endonuclease analysis of Ad5denV and the plasmids used in its construction. Viral DNA and plasmids were digested and run on an 0.8% agarose gel. (A) Lanes 1–8 show the ethidium bromide-stained gel. (B) Lanes 2–8 show the Southern blot probed with the 1.9 kb *denV* cartridge. The probe used was the 1.9 kb *Xba*I fragment which was gel isolated from an *Xba*I digest of pEMBL18/RSVdenV. Two hundred nanograms of the fragment was nick translated to a sp. act. of $\sim 2 \times 10^9$ c.p.m./μg. The marker used was bacteriophage lambda DNA digested with *Hin*dIII which produces bands of 23 130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp. Ethidium bromide-stained marker lanes are shown in lanes A1 and B1 for comparison purposes. Lane 2 contains Ad5dl309 digested with *Hin*dIII; lane 3 contains ad5denV digested with *Hin*dIII; lane 4 contains pMCADV digested with *Hin*dIII; lane 5 contains Ad5den V digested with *Xba*I; lane 6 contains pMCADV digested with *Xba*I; lane 7 contains pEMBL18/RSVdenV digested with *Xba*I; and lane 8 contains pFGDX1 digested with *Hin*dIII.

(5'-CGCGGCAATTCACGATATTCAGCC-3') corresponding to the complementary sequence from positions 668 to 645 (inclusive) in the *denV* expression cartridge (Figure 2). RNA was extracted from Ad5 infected HeLa cells by the method of Berk and Sharp (49) and primer extension reactions were carried out as described by Jones *et al.* (50).

Irradiation of virus

UV irradiation of virus was performed using a germicidal lamp (General Electric Germicidal Lamp G8T5) emitting a wavelength of predominantly 254 nm. The method employed was essentially the same as that described previously (31). Stock virus was diluted at least 10-fold with cold alpha-MEM without serum and a 1 ml aliquot of virus suspension was irradiated in a 35 mm diameter Petri dish (Falcon Plastics) with the dishcover removed, kept on ice, with constant swirling during the irradiation. Under these conditions the incident dose rate was in the range of 1–2 J/m^2/s as determined using a J-225 shortwave UV meter (Ultraviolet Products, San Gabriel, CA).

Assay for survival of adenovirus Vag expression

Non-irradiated and irradiated suspensions of Ad2 were assayed for their abilities to form viral structural antigens (Vag) in the different human fibroblast strains as described previously (30). Ad2 is a double-stranded DNA virus which replicates in the nucleus of a susceptible host cell, forming large quantities of viral structural proteins (51) which can be readily detected by immunofluorescent staining. The expression of viral genes is ordered during the adenovirus infectious cycle, and viral DNA synthesis is a requirement for the expression of Vag proteins (52).

Fibroblast monolayers grown in 8 well chamber slides (Lab Tek Products, Naperville, IL) were infected with either irradiated or non-irradiated Ad2. Three serial dilutions of the virus were used to infect each slide. Duplicate wells were used for each viral dilution, with the two additional wells serving as uninfected controls. The dilution series for non-irradiated and irradiated virus were prepared separately, non-irradiated virus being diluted to a greater extent than the irradiated virus. Following viral adsorption for 90 min, infected cells were incubated in growth medium. At 48 h after infection, the monolayers were fixed in cold acetone/ethanol mixture (1:1), incubated in the presence of rabbit Ad2 antiserum for 30 min at 37°C, and then incubated for 30 min with fluorescein-conjugated anti-rabbit globulin. For each slide, the number of fluorescing centres was counted in duplicate wells at three serial dilutions of the virus, and the data points were fitted to a straight line using least-squares analysis. Taking into account the dilution factor, the slope of the line was used as a quantative measure of Vag formation. Survival of this viral function was then taken as the ratio of Vag formation for irradiated virus compared to that for non-irradiated virus.

Results

Construction of an Ad5 recombinant virus encoding the denV gene of bacteriophage T4

The use of adenovirus as a vector to deliver a foreign gene into mammalian cells was demonstrated by Haj-Ahmad and Graham (35) and a number of adenovirus vectors have been constructed that express various foreign genes (35–39). A method similar to that used by Haj-Ahmad and Graham (35) was used to construct a recombinant adenovirus vector carrying the *denV* gene from bacteriophage T4.

The outline for construction of Ad5denV is shown in Figure 1. Firstly, plasmid pEMBL18/RSV(denV) was constructed as described in Materials and methods and subsequently digested with *Xba*I to release the *denV* expression cartridge as shown in Figure 2. The *denV* expression cartridge was a 1.9 kb fragment containing the RSV LTR promoter and *denV* sequences plus the SV40 large T splice and polyadenylation signals. This *Xba*I fragment was gel isolated, extracted and ligated into the *Xba*I site of pFGDX1. The resulting pBR322-based plasmid, pMCADV, contains the right 40% of the Ad5 genome with the denV cartridge inserted into the E3 region of Ad5. pMCADV was then cleaved with *Sal*I to linearize it, and co-transfected with *Eco*RI-digested Ad5dl309 into human 293 cells using the calcium chloride method (46). *In vivo* recombination between overlapping homologous regions of the Ad5dl309 *Eco*RI A fragment and pMCADV ($\sim$16% of the Ad genome) resulted in the formation of Ad5denV.

Figure 3 shows the digestion (Figure 3A) and Southern blot (Figure 3B) analysis of vectors involved in the construction of Ad5denV. The blot was probed with the 1.9 kb *denV* expression cartridge obtained by digestion of pEMBL18/RSVdenV with *Xba*I and subsequent gel isolation and nick translation. In lane 7 the parental plasmid pEMBL18/RSVdenV digested with *Xba*I can be seen to release the 1.9 kb fragment containing the *denV* gene,

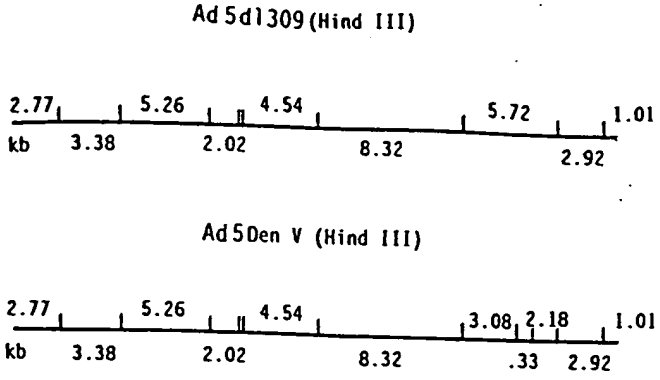

Fig. 4. *Hind*III digestion patterns of Ad5dl309 and Ad5denV. Shows fragment sizes in kilobase pairs.

leaving the 5.6 kb remainder containing only the pEMBL18 sequences. pFGDX1 is shown in lane 8 digested with *Hind*III. producing bands of 9.1, 3.54, 2.9 and 1.34 kb. The *Hind*III digestion pattern of pMCADV is show in lane 4. Comparison with the restriction pattern of pFGDX1 shows the pMCADV has lost the 3.54 kb fragment (which contains the *Xba*I site) and has additional bands of 3.1, 2.2 and 0.33 kb, due to the two internal *Hind*III sites in the *denV* containing cartridge. All three additional bands contain sequences specific to the *denV* cartridge as illustrated in the Southern blot (Figure 3B). The orientation of the insert can be determined from the known positions of the *Hind*III sites within the cartridge (Figure 1). The *Hind*III restriction fragment sizes obtained for pMCADV and Ad5denV indicate that the cartridge is inserted with the RSV promoter transcribing from right to left along the Ad genome, and this orientation is depicted in Figures 1 and 4. Confirmation of transfer of the *denV* cartridge is Ad5denV is shown by the *Hind*III and *Xba*I restriction digestion patterns of Ad5denV shown in lanes 3 and 5 respectively. These can be compared with *Hind*III and *Xba*I digests of the source plasmid pMCADV shown in lanes 4 and 6 respectively, as well as a *Hind*III digest of the parental virus Ad5dl309 shown in lane 2. The disappearance of the Ad5dl309 *Hind*III B fragment and the addition of the *denV*-specific 3.1, 2.2 and 0.33 kb bands in lane 3 confirm the construction of the desired recombinant Ad5denV.

RNA transcription of denV-specific sequences in Ad5denV-infected cells

Previous studies have utilized the detection of correctly initiated RNA transcripts as an indication of gene expression in virally infected cells (53), and this strategy was employed in order to examine expression of *denV*-specific transcripts from Ad5denV-infected cells. The technique of primer extension (50) was performed using RNA extracted from Ad5denV-infected HeLa cells at 1 h and 22 h post-infection. Cells were infected at 30 plaque forming units/cell and 30 μg of each RNA sample was used for each extension assay. The 24mer oligonucleotide synthesized was homologous to the *denV* expression cartridge, complementary to positions 668−645 (inclusive) on the denV cartridge (Figure 2). It can be seen in Figure 5 that while no appreciable background radioactivity was observed in either the purified primer (lane 1) or in the reaction with uninfected HeLa cell RNA (lane 5), or HeLa cell RNA extracted 1 h after infection with Ad denV (lane 3), a strong radioactive signal appears at ~ 124 bases for the HeLa cell RNA extracted 22 h after infection with Ad5denV (lane 4). The strong radioactive signal indicates an abundant RNA transcript that has been initiated at position

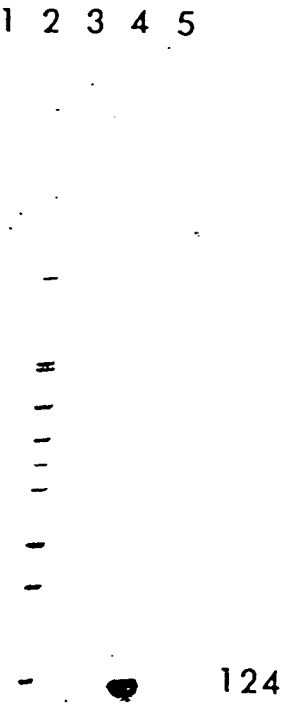

Fig. 5. Primer extension of Ad5denV-infected HeLa cell RNA. A 24 base oliognucleotide corresponding to the complementary strand of the *denV* cartridge sequence from position 668 to 645 (inclusive) was kinased and used as a primer for reverse transcription of total cellular RNA harvested from AD5denV-infected and non-infected HeLa cells. Lane 1 contains the input primer. Lane 2 contains size markers of *Hpa*II-digested pBR322, Klenowed with radiolabelled nucleotides, and strand separated. Sizes of fragments are 623, 528, 405, 310, 243, 239, 218, 202, 191, 181, 161, 161, 148, 148, 124, 111, 91, 77, 68, 35, 35, 27, 27, 16, 10 and 10 bases. Lanes 3 and 4 show the products of reverse transcription of RNA samples from Ad5denV-infected HeLa cells harvested at 1 h and 22 h post-infection respectively. Lane 5 shows the products of reverse transcription of the RNA sample from uninfected HeLa cells.

545 in the cartridge sequence, which corresponds to published reports for the start of transcription of the RSV 3′ LTR promoter. This result shows that properly initiated *denV*-specific RNA was made in Ad5denV-infected HeLa cells.

Survival of UV-irradiated Ad denV in human fibroblasts

Previous reports have shown that the introduction of the denV protein into XP group A cells by membrane permeabilization using Sendai virus (17), and by transfection of the *denV* gene (23,24), can partially restore the DNA capacity as measured by increased cell survival after UV irradiaton. Since the survival of Vag formation for UV-irradiated Ad is reduced in XP compared to normal human fibroblasts (30), it was considered of interest to examine the survival of UV-irradiated Ad5denV following the infection of XP as well as normal human fibroblasts.

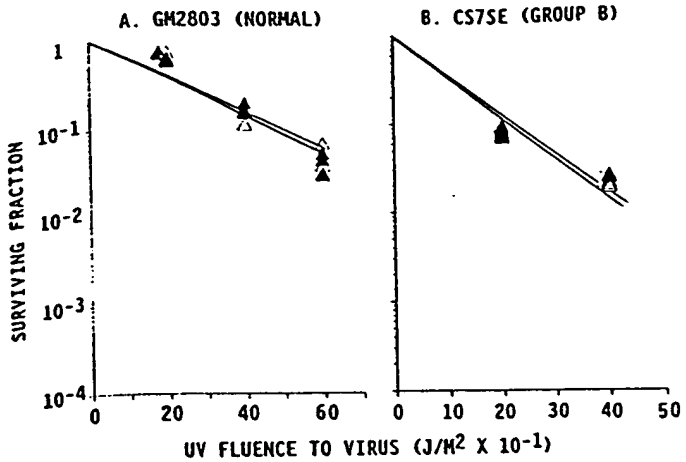

Fig. 6. Survival of Vag formation for UV-irradiated Ad5denV and Ad5VSV after infection of normal and Cockayne syndrome fibroblasts. Pooled results for a number of experiments using (A) normal fibroblast strain GM2803 and (B) Cockayne syndrome fibroblast strain CS7SE. Ad5denV, closed symbols and Ad5VSV, open symbols. The number of experiments for each cell strain is shown in Table I. Error bars for the survival points of individual experiments are within the symbol size and the survival curves are drawn using the D_0 values of Table I.

Unirradiated and UV-irradiated suspensions of Ad5denV and Ad5VSV were assayed for their ability to form Vag in a number of different human fibroblast strains, including two normal strains, a Cockayne syndrome (CS) strain and three unrelated excision defficient XP strains from complementation groups A, C and E. It can be seen from Figure 6 that the survival of Vag formation for UV-irradiated Ad5VSV was similar to that for Ad5denV after infection of the normal strain GM2803 and the CS strain CS7SE from complementation group B. In contrast, survival of Vag formation for UV-irradiated Ad5denV was greater than that for UV-irradiated Ad5VSV after infection of the three XP strains, as shown in Figure 7. These results indicate a partial complementation of the DNA excision repair deficiency in XP cells as a result of expression of the *denV* gene encoded in Ad5denV.

UV survival curves for Ad Vag formation in the normal and CS strains were consistent with simple exponential inactivation over the range of doses employed and D_0 values calculated using linear regression analysis for this single component are shown in Table I. UV survival curves for Vag formation of Ad5VSV and Ad5denV showed two components for infection of the three XP strains as has been reported previously for the

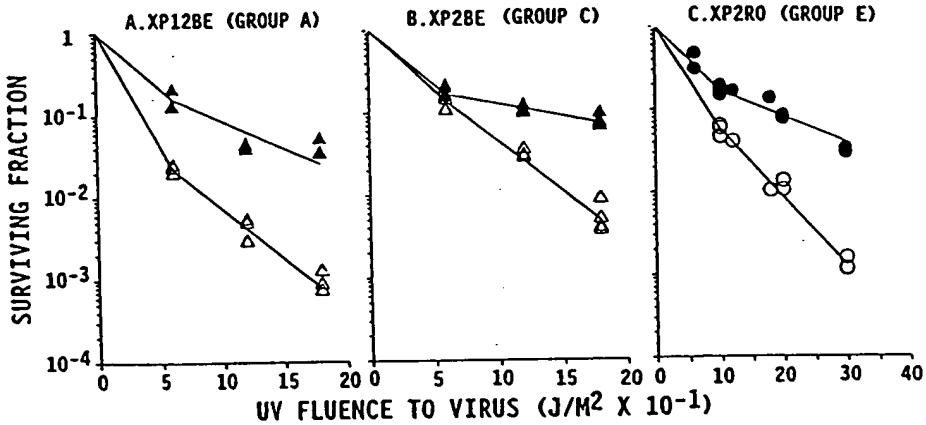

Fig. 7. Survival of Vag formation for UV-irradiated Ad5denV and Ad5VSV after infection for XP fibroblasts. Pooled results for a number of experiments using (A) XP12BE (group A), (B) XP2BE (group C) and (C) XP2RO (group E). Ad5denV, closed symbols and Ad5VSV, open symbols. The number of experiments for each cell strain is shown in Table I. Error bars for the survival points of individual experiments are within the symbol size and the survival curves are drawn using the D_0 values of Table I.

Table I. UV survival of Ad5denV and Ad5VSV

Virus	Cell strain	First component		Second component		No. of exp.
		D_0	% HCR	D_0	% HCR	
Ad5VSV	Normal					
	GM2803	175 ± 12				4
	B.H.	206 ± 10				3
	Mean	190.5				
	Xeroderma pigementosum					
	XP12BE (A)	16 ± 1	8.4	38 ± 2	19.9	3
	XP2BE (C)	30 ± 2	15.7	36 ± 2	18.9	4
	XP2RO (E)	28 ± 5	14.7	53 ± 3	27.8	4
	Cockayne syndrome					
	CS7SE (B)	109 ± 8	57.2			3
Ad5denV	Normal					
	GM2803	178 ± 13				4
	B.H.	207 ± 13				3
	Mean	192.5				
	Xeroderma pigmentosum					
	XP12BE (A)	35 ± 3	18.2	79 ± 18	41.0	3
	XP2BE (C)	34 ± 2	17.7	156 ± 24	81.0	4
	XP2RO (E)	60 ± 5	31.2	199 ± 9	103.4	4
	Cockayne syndrome					
	CS7SE (B)	103 ± 6	53.5			3

survival of Ad2 in these and other XP strains (30–32). D_0 values for both components of each curve are shown in Table I. The D_0 for the first component calculated in this way could be an overestimate since the regression analysis was based on the single, lowest UV exposure survival data points of each curve.

The D_0 values obtained for infection of the CS and XP strains were expressed as a percentage of the average value obtained for the two normal strains. Percentage of host cell reactivation values for Vag formation of UV-irradiated Ad were taken as measure for the repair capacity of the infected cells and are shown in Table I. The degree of restoration in UV survival for Ad5denV compared to Ad5VSV following infection of the XP strains was different for each of the XP strains tested. The restoration in repair capacity due to the presence of the *denV* gene was greatest for the XP group E strain, where the second component of the Ad5denV survival curve reached a level similar to that found after infection of normal fibroblasts.

Discussion

The results of this work show a partial complementation of the DNA excision repair deficiency in primary XP fibroblasts from complementation groups A, C and E as a result of the expression of the *denV* gene from bacteriophage T4 encoded in Ad5denV.

UV survival of Vag formation for Ad5VSV in the various human fibroblasts tested was similar to that previously reported for Ad2 (30–31). UV survival of Ad5VSV was similar to that of Ad5denV after infection of the CS strain, indicating that the DNA repair deficiency of the CS strain is not complemented by the *denV* gene product. This is consistent with other reports which indicate that CS cells are not deficient in the incision step of excision repair following UV damage (54,55). Differences in the degree of restoration for the survival of UV-irradiated Ad5denV in the three XP fibroblast strains tested are thought to reflect differences in the nature of the DNA repair deficiency found in the different XP complementation groups tested.

Survival curves for UV-irradiated Ad following the infection of XP cells were consistent with a two-component nature as reported previously (30,32), suggesting two components for the repair of UV-induced DNA damage in these cells. UV survival curves for some XP cells themselves also show a two-component nature (56,57), which may also reflect two components for the repair of UV damage in these cells. However, it is not known whether the two-component nature of the survival curves for UV-irradiated Ad in XP cells and the two-component nature of the UV survival curves of the cells themselves are a reflection of the same two repair processes. Recent work showing an increased UV sensitivity following the addition of aphidicolin to normal CS and XP cells gives good evidence for the existence of two pathways for the excision repair of UV-damaged DNA in human fibroblasts (57). While it has been suggested that the two independent pathways arise from independent polymerase action (57), current data on the role of secondary structure in the repair of DNA (58) suggests that access to the DNA damage could also be of fundamental importance in the excision repair process.

In previous studies the cloned *denV* gene has been introduced into SV40-transformed XP12RO(M1) cells from complementation group A (23) as well as an XP group D–HeLa hybrid cell line (24) using DNA-mediated transfection. The stable transformants so produced show partial restoration of colony-forming ability and excision repair synthesis after UV irradiation. For the SV40-transformed XP group A cells, partial restoration in cell survival after UV was of a similar magnitude for the partial restoration in Ad Vag survival after UV seen here following infection of the XP group A fibroblasts. The fact that restoration by *denV* is only partial in XP cells may reflect one or more characteristics of the *denV* gene product when introduced into human cells. Firstly, the *denV* protein recognizes and initiates the repair of UV-induced pyrimidine dimers, while leaving behind other UV-induced lethal lesions (23). This is in agreement with reports concerning the biological effects of pyrimidine dimers and [6–4] pyrimidine–pyrimidone photoproducts, neither of which are efficiently removed in XP cells (10–13). Secondly, not only does the action of *denV* leave non-dimer photoproducts, it also leaves a modified cyclobutane dimer with an unusual pair of termini that are not normally observed in human cells (59,60). Hence the partial restoration in XP cells may be due to that fact that the cell still has to repair a modified dimer. It is also possible that the partial restoration of repair in XP cells by *denV* reflects incomplete cutting at cyclobutane dimers due to inaccessibility in chromatin of the denV protein. Therefore, the increase in survival of UV-irradiated Ad due to the *denV* gene when infecting XP cells may not be a true reflection of the role of pyrimidine dimers in the viral DNA.

The results of this work demonstrate that an adenovirus vector can be effectively used to study the expression of DNA repair genes in a variety of untransformed mammalian cell types. The use of the 3′ LTR promoter from RSV appears to be an effective choice of eukaryotic promoter since abundant transcription was found to occur from this promoter following infection by Ad5denV. We are presently constructing an E1-deleted mutant of Ad5denV that can be grown in human 293 cells (35,40) but results in non-lytic infection of human fibroblasts. Infection of primary human fibroblasts with such a vector would allow an examination of the effects of the *denV* gene product on the UV survival of XP fibroblasts themselves. If successful, this approach could be used to examine the expression of other cloned DNA repair genes in primary human cells.

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Simian Virus 40 Small-t Does Not Transactivate RNA Polymerase I Promoters in Virus Infections

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Transcriptional stimulatory properties of virus-encoded transactivators appear to be critical for viral gene expression and may be linked to cellular transformation in certain cases. Recently, the simian virus 40 (SV40) 17-kDa small-t antigen was shown to stimulate transcription of polymerase II and III genes in transient transfection assays. In experiments performed in our laboratory, two of the polymerase II promoters f the adenovirus genome, namely, the EII-early and EIII promoters, were transactivated by the small-t antigen in transient transfection assays. To further elucidate the mechanism of this transactivation, we examined the ability of small-t to transactivate the adenovirus type 5 EII-early and EIII promoters in CV-1 cells under conditions in which the small-t gene or the reporter genes were introduced into the cells through transfection and other routes. In one approach, we used established CV-1 cell lines which constitutively express the small-t gene, and study of the EII-early promoter was afforded by infection of an EIA-negative adenovirus type 5 variant. For the second approach, a recombinant adenovirus was constructed in which small-t was expressed from a replication origin-negative SV40 early promoter in the EIA region of an adenovirus vector (Ad-SV-t). The effect of small-t on adenovirus EII-early and EIII promoter expression was studied in coinfection or single-infection experiments. In both cases, transcription of the adenovirus early promoters was not stimulated by small-t. These and other results indicate that transactivation of polymerase II promoters by small-t occurs only when the target gene is in a transiently transfected state. Thus, small-t-mediated transactivation of polymerase II promoters is dependent on the type of assay system used and may be mechanistically different from that of the widely studied EIA.

: past decade has brought to light a number of virus-encoded transcriptional transactivators that appear to regulate the temporal expression of virus-encoded genes and in some instances even affect the expression of the genes of the host cell. The oncogenic potential of some of these viruses is probably an attribute of the latter property of the transactivators. Among the better-studied examples of viral transactivators are the adenovirus (Ad) E1A 289-amino-acid polypeptide (33), Ad EIV 19.5-kDa polypeptide (20, 24), simian virus 40 (SV40) large-T antigen (7), Tax proteins of human ·ll leukemia virus types I (25) and II (9), Tat protein of i· nan immunodeficiency virus (11), hepatitis B virus X protein (27), and herpes simplex virus ICP0 (41) and VP16 (48) proteins. In Ad infection of human cells, the 289-amino-acid E1A protein is known to coordinately regulate the efficient expression of the six early RNA polymerase II promoters of the virus in addition to the single RNA polymerase III promoter (33). In addition, EIA has been shown to transactivate several cellular genes such as the human hsp70 (32) and tubulin (43) genes and the oncogene c-*myc* ·22). The SV40 large-T antigen is known to bind to its own ·ly and late promoters with variable effects; i.e., it re-·esses transcription from the former and stimulates it from the latter (7, 28). It also binds some host promoters rather promiscuously and acts as a regulator of transcription (13, 23, 40). Hepatitis B virus is regarded as the main etiologic factor in the development of human hepatocellular carcinoma. It has been shown that the X gene codes for a transactivating function, and it is able to induce both polymerase II and III promoters (49). The 15.5-kDa human immunodeficiency virus Tat protein is a potent transactiva tor of the expression of all sequences linked to the huma immunodeficiency virus long terminal repeat (11).

Recently Loeken et al. showed that in transient transfec tion assays, SV40 small-t enhances expression of the A EII-early promoter and the VAI RNA gene (29). This wa the first occasion in which a specific function seemed to b attributable to this enigmatic protein. To determine th mechanism of small-t-mediated transactivation of polyme ase II promoters, we have studied the effect of small-t on A EII-early promoter expression in cells in which small-t wa introduced into cells by two routes other than transfectio In one set of experiments, we used cell lines which const tutively express the small-t gene, while in the second set c experiments we used a novel EIA-negative Ad vector th expresses the small-t gene in large amounts. Our resul indicate that the small-t protein does not stimulate transcri tion of the EII-early promoter in either of these experiment approaches. In agreement with earlier reports, we do see a efficient stimulation of Ad EII-early and EIII promoters i response to small-t in transient assays. These and oth results lead us to suggest that the transactivation of th polymerase II promoters by small-t is dependent on the typ of assay system used and may be mechanistically differe from that of the widely studied EIA.

MATERIALS AND METHODS

Cells, plasmids, and viruses. HeLa, human 293, and CV cells were maintained in Dulbecco modified Eagle mediu containing 10% calf serum for the two former cell lines an 10% fetal calf serum f r CV-1 cells. Two established ce lines used in these studies are CV-1/t18 and CV-1/t26, CV

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cells which contain and constitutively express the SV40 small-t gene. These cell lines were constructed as described previously for mouse cells (35). Plasmid pW2t/cDNA (35) is a small-t clone that contains cDNA sequences coding for the SV40 small-t gene fused to the SV40 early promoter and contains SV40 sequences from 294 (*Kpn*I site) to 2533 (*Bam*HI site; numbering as in reference 8). The SV40 sequences in this plasmid are in the pGEM background. The *Hpa*I fragment from 2666 to 3733 of the SV40 genome is deleted in pW2t. Plasmid pCD2 contains 0 to 5.0 map units (m.u.) of the Ad5 genome. In this plasmid, Ad5 DNA sequences from 0 to 5.0 m.u. (*Sst*II site) were cloned between *Bam*HI and *Eco*RI sites of pBR322 after appropriate modification of the vector (31). Plasmid pEII-early CAT contains coding sequences for the reporter chloramphenicol acetyltransferase (CAT) gene fused to the Ad EII-early promoter (31).

Adenovirus mutant *dl*309 is a phenotypically wild type Ad5 variant that has a single *Xba*I site at 4.0 m.u. (46). Mutant *dl*321 is an EIA-negative virus that contains the same deletion as *dl*312 (30). Mutant *dl*312 lacks EIA coding sequences from 1.0 to 4.0 m.u. (26). Construction of the Ad mutant, which contains the CAT gene under the control of the wild-type EII-early promoter (Ad-EII-E-CAT), has been described in a previous report (30). This is an EIA-negative virus that contains the EII-early-CAT gene in its EIII region. Direction of transcription of the EII-early-CAT gene in this virus is the same as that of the resident EII gene.

Construction of Ad-SV-t. The recombinant Ad, Ad-SV-t, was constructed as follows: (i) the *Pvu*II site at Ad position 452 of pCD2 was first converted into a *Kpn*I site; (ii) the SV40 replication origin (ori) of pW2t was converted to ori$^-$ by transferring an appropriate DNA fragment from a plasmid that contains an ori$^-$ mutation; (iii) the small-t cDNA sequences along with the SV40 early promoter were then inserted between the *Kpn*I and *Bam*HI sites of a polylinker-containing vector; (iv) taking advantage of the *Xba*I site immediately downstream of the *Bam*HI site, the *Kpn*I-to-*Xba*I fragment, which contains the small-t gene, was cloned between *Kpn*I and *Xba*I sites of the modified pCD2 (pPR108); and (v) the recombinant Ad was then constructed by ligating the 0- to 4.0-m.u. fragment from pPR108 to the 4.0- to 100.0-m.u. fragment of *dl*309 and transfecting 293 cells with the ligated DNA sample as described by Stow (44). The virus was plaque purified twice, and the variant was again confirmed by restriction endonuclease digestion of the viral DNA.

CAT assays. Plasmid transfections or viral infections were carried out as indicated in the figure legends, in the absence or presence of cytosine arabinoside (ara-C) (25 μg/ml). The cells were harvested at the time points indicated in the figure legends, and cell lysates were prepared by sonication. Protein concentrations were determined by the method of Bradford (6), and all assays were carried out with equal amounts of proteins. CAT activity was assayed as described previously (18). Assays were performed under conditions in which conversion of [^{14}C]chloramphenicol to its products was less than 30% of the total substrate added. When necessary, the cell lysates were diluted to obtain values within this range. Further quantitation was afforded by liquid scintillation counting of the converted and unconverted spots on the thin-layer chromatogram. Transfection efficiency was monitored by including an expression plasmid that expresses a secreted form of the bacterial alkaline phosphatase gene and measuring alkaline phosphatase secreted into the medium (2).

Immunoprecipitations. HeLa or CV-1 cells were infected with the Ad-SV-t virus, and the infection was allowed to proceed for 8, 16, or 24 h. Cells were labeled for the last 8 h of the infection with 200 μCi of [^{35}S]methionine per ml, and cytoplasmic extracts f the cells were prepared with Triton X-100 (20 mM Tris [pH 8.0], 150 mM NaCl, 0.2% Triton X-100). Immunoprecipitations were performed with a hamster polyclonal serum that had been generated against the SV40 tumor antigens. Extracts prepared from 2.5×10^5 cells were incubated with the antibody for 45 min at 4°C, and the complex was incubated with *Staphylococcus* protein A for a further 15 min on ice. The immune complexes were denatured with sodium dodecyl sulfate (SDS) and analyzed on 20% SDS-polyacrylamide gels as described previously (45). The protein bands were visualized by fluorography.

Primer extension analysis. CV-1 or HeLa cells were infected with *dl*309 (EIA$^+$), *dl*321 (EIA$^-$), or Ad-SV-t at 25 PFU per cell, and the infection was allowed to proceed for 8 or 16 h in the presence of 25 μg of ara-C per ml. Total cytoplasmic RNA was prepared as described by Sambrook et al. (38). Thirty to 50 μg of the total RNA was used in each primer extension assay. The assay was performed as described in reference 1 except for the following details. The Ad EIII primer was complementary to +108 to +133 (with +1 as the RNA start site), whereas the β-actin primer used for normalization of the amount of RNA was complementary to sequences extending from +78 to +105 of the human β-actin gene. Primers were end labeled at the 5′ end, 10^5 cpm was used per extension reaction, and the annealing was carried out overnight at 30°C, as described previously (1). After precipitation of the nucleic acids, the annealed primer was extended for 3 h at 42°C with 40 U of avian myeloblastosis virus reverse transcriptase (Promega Biochemicals), and the extended products were analyzed on 6% DNA-sequencing gels.

RESULTS

SV40 small-t transactivates Ad EII-early and EIII promoters in transfection assays. To determine whether SV40 small-t stimulates transcription of RNA polymerase II promoters in our experiments, we performed standard transfection assays in which the small-t plasmid was cotransfected with an EII-early or EIII CAT plasmid (in which CAT gene expression is controlled by the Ad EII-early or EIII promoter) into CV-1 cells by the calcium phosphate precipitation method. Transfection efficiency was monitored by including an expression plasmid that encodes a secreted form of the bacterial alkaline phosphatase gene (2). CAT activity was then estimated in the lysates as described previously (18). In the experiment shown in Fig. 1, E1A stimulated EII-early and EIII promoters by 30-fold, whereas small-t stimulated EII-early and EIII promoters by 35- and 10-fold respectively.

In a recent report by Herrmann and Mathews (21), the Ad EIB 19-kDa protein was shown to increase the expression of a polymerase II gene in similar transfection assays. Further analysis showed that the cause for this transactivation by the 19-kDa protein was due to increased stabilization of the reporter gene in the cells rather than transcriptional transactivation of the gene by the 19-kDa protein. To rule out the possibility of a similar effect in our small-t cotransfection experiments, we quantitated the CAT plasmid up to 96 h posttransfection in mock, EIA, and small-t cotransfections in duplicate sets of experiments with dot blot hybridizati ns (21). Our results showed that small-t did not stabilize EII-

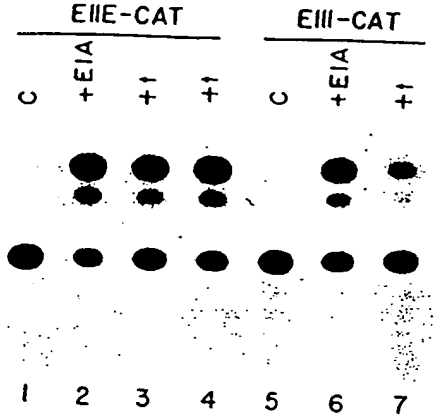

FIG. 1. Transactivation of the Ad EII-early and EIII promoters by small-t or EIA in transient transfection assays in CV-1 cells. Two micrograms of the EII-early CAT plasmid or 10 μg of the EIII-CAT plasmid was cotransfected into 35-mm dishes with 2 μg of the pGEM (control) (lane C), EIA (pCD2) (lane +EIA), or small-t (pW2t) (lane +t) plasmid by the standard calcium phosphate precipitation method. An expression plasmid which expresses a secreted form of bacterial alkaline phosphatase gene was used as an internal control to measure transfection efficiency; 225 μg of protein was used for CAT assays. To quantitate CAT activity, samples in lanes 2, 3, 6, and 7 were diluted 10-fold and reassayed (not shown).

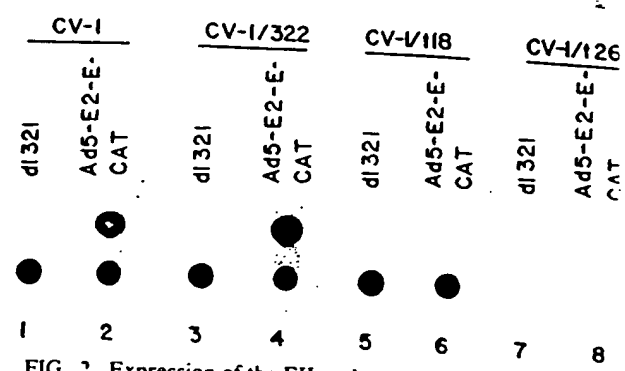

FIG. 2. Expression of the EII-early promoter in stable CV-1 c lines that constitutively express small-t. The EII-early promo expression was assayed by infecting the cells with a virus whi contains EII-early CAT in the EIII region (Ad-EII-E-CAT [31 Cells were infected with Ad variants at 25 PFU per cell for 8 h a harvested, and CAT activity was assayed as described previous (18). Ad5-E2-E-CAT is an E1A-negative virus in which EIII substituted with EII-early CAT construct (30). *dl*321 is an EI negative Ad variant. CV-1/t18 and CV-1/t26 are two stable CV-1 c lines which express small-t. CV-1/322 is a control G418-resistant c line.

early CAT plasmid, indicating that increased CAT activity was not due to the stabilization effect (data not shown).

Small-t expressed from constructed cell lines does not transactivate the Ad EII-early promoter. To determine the transactivation potential of the small-t antigen in systems that do not involve transfection, we used cell lines and viruses that express the protein. Phillips and Rundell (35) have described the construction of mouse C3H 10T1/2 cell lines that constitutively express the small-t antigen of SV40. These cell lines were obtained by cotransfection of pSV2-neo, and plasmids that encode the small-t and were selected for by G418 resistance. CV-1 cell lines that express small-t antigen were made in a similar fashion. These cell lines express small-t at levels comparable to those found in COS cells (17). To determine whether small-t synthesized by these cells transactivates the EII-early promoter, cells were infected with an EIA-negative Ad variant in which the EIII region is substituted by the EII-early-CAT gene (Ad EII-E-CAT [31]). We showed earlier that the newly introduced EII-early promoter in this virus was efficiently transcribed and transactivated by EIA when coinfected with wild-type Ad (30). Cells were infected with Ad EII-E-CAT at 20 PFU per cell for 8 h and harvested, and CAT activity was determined in cell lysates. As shown in Fig. 2, there was no stimulation of CAT activity by small-t in these cell lines compared with a control G418-resistant cell line (35). Rather, for reasons that we cannot explain at present, we consistently observed a threefold decrease in EII-early promoter expression in these cells. These cells also did not stimulate EII-early promoter when the promoter was introduced into cells by transient transfection (data not shown). To rule out the possibility that the decreased CAT activity in the small-t cell lines was due to differences in infection efficiency, the cells were infected with wild-type Ad5, and the expression of late proteins was monitored at 20 h postinfection. The levels of protein in the control and the small-t-expressing cell lines were found to be comparable to that of a CV-1 cell infection (data not shown).

Small-t expressed from a recombinant Ad does not transactivate the Ad EII-early and EIII promoters. Because we did not see any transactivation in the experiments performed on the small-t cell lines, we tried a different approach to address the same question. We constructed an EIA-negative Ad vector in which small-t was expressed from the SV40 early promoter. The cDNA copy of the small-t gene along with its promoter was cloned into the Ad5 variant *dl*309 between nucleotides 422 and 1336, as described in Materials and Methods. The structure of this recombinant Ad (Ad-SV-t) is illustrated in Fig. 3.

To determine whether Ad-SV-t synthesized small-t in appreciable amounts, CV-1 cells were infected with Ad-SV-t at 25 PFU per cell, and immunoprecipitations were performed as described in Materials and Methods. Substantial quantities of small-t were detected at 16 h postinfection (Fig. 4). Similar results were obtained in HeLa cells (data not shown).

To determine the transactivation potential of the virus Ad-SV-t, CV-1 cells were coinfected with Ad-SV-t and Ad EII-E-CAT at 25 PFU per cell. Mutant *dl*309 (EIA$^+$) was used as a positive control. Cells were harvested 8, 16, and 24 h after infection, and the CAT activity present in the lysates was determined. The EII-early promoter activity was in-

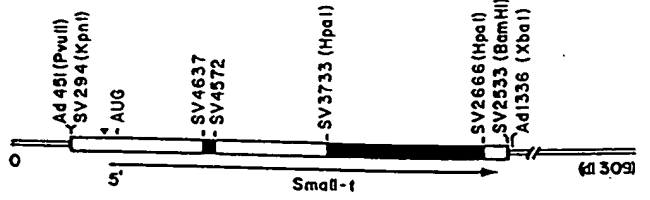

FIG. 3. Structure of the recombinant Ad (Ad-SV-t), which expresses the SV40 small-t antigen. The map units are positioned on the Ad chromosome; the numbers refer to nucleotides on the Ad (16) or SV40 (8) chromosome. Construction of the virus is described in Materials and Methods. The solid block shows deletions. The inverted triangle shows a 6-bp insertion at the replication origin (*Bgl*I site) of the SV40 DNA. The solid line with an arrow below the viral chromosome shows the primary transcript synthesized from the small-t gene.

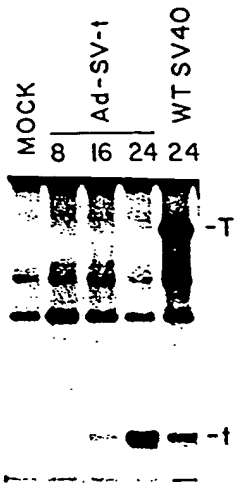

FIG. 4. Immunoprecipitation of small-t from CV-1 cells infected with Ad-SV-t. CV-1 cells were infected with the Ad-SV-t variant for 8, 16, or 24 h at 25 PFU per cell, and the cells were metabolically labeled with 200 μCi of [^{35}S]methionine per ml for the last 8 h of infection. Small-t was immunoprecipitated with a polyclonal antiserum as described in Materials and Methods, and the proteins were analyzed on a 20% SDS-polyacrylamide gel. Mock- and SV40-infected cells were also immunoprecipitated with the same antibody simultaneously. WT, wild type.

creased by EIA by about 11-fold at 16 h postinfection, whereas small-t failed to induce this promoter at all time points tested (8, 16, or 24 h postinfection; Fig. 5A and Table 1), and the extent of stimulation was less than twofold that produced by our EIA$^-$ control in any of our numerous experiments. At 24 h postinfection, most of the cells come off the plate in *dl*309 infections. This is probably why we did not see transactivation of the EII-early promoter by *dl*309 at 24 h after infection (Table 1).

Treatment of infected cells with ara-C for prolonged periods lengthens the early phase of infection and induces EIA proteins to high levels, leading to increased stimulation of viral EIA-sensitive promoters (15). To determine whether ara-C treatment would facilitate the small-t-mediated transactivation of the EII-early promoter, we performed coinfection experiments in the presence of ara-C. Although the EIA-mediated transactivation of the EII-early promoter was increased in these experiments, ara-C treatment did not improve that of small-t (Fig. 5B and Table 1). Finally, because small-t was expressed from a human Ad vector that replicates in human cells, we examined whether small-t expressed from Ad-SV-t would transactivate EII-early promoter in human cells. HeLa cells were infected with Ad-SV-t and Ad EII-E-CAT for 8 and 16 h, and the CAT activity expressed from the chimeric gene was determined. As shown in Fig. 5C, small-t failed to transactivate the EII-early promoter, whereas EIA transactivated the EII-early promoter efficiently. These results lead us to conclude that the SV40 small-t does not transactivate the Ad EII-early promoter in a manner similar to that of the Ad EIA.

As was mentioned previously, the SV40 small-t gene in our construct is under the control of the SV40 early promoter/enhancer sequences. Since the regulation of SV40 gene expression is on a slower temporal scale than is Ad gene expression, we considered the possibility that the difference in transactivation of the EII-early promoter by EIA and small-t may be due to the difference in levels of the proteins in the cell for a given time point. To rule out this possibility, we performed preinfection experiments in which the transactivator virus (containing either EIA or small-t) was infected into CV-1 cells, and the reporter EII-early CAT virus was superinfected into these cells 8, 16, or 24 h after the first infection. The double infection was then allowed to proceed for a further 8 h. Transcription from the EII-early promoter was estimated by assaying for CAT activity in cell lysates of these infected cells. There was no appreciable increase in expression of the CAT gene even when small-t was allowed to accumulate in the cell for 16 h (data not shown).

The EII-early promoter is rapidly activated in the Ad growth cycle. Small-t in our Ad-SV-t virus was expressed from the SV40 early promoter, which is activated more slowly in natural SV40 infection than is an Ad early promoter. It was therefore possible that small-t did not accumulate rapidly enough to cope with the kinetics of the Ad EII-early promoter activation. To rule out this possibility, we constructed another Ad variant, Ad-CMV-t, in which the SV40 small-t was expressed from the enhancer/promoter of the major immediate-early gene of human cytomegalovirus (51). Expression of the small-t from this extremely strong promoter resulted in the accumulation of substantial amounts of protein 6 to 8 h after infection (comparable with that produced by Ad-SV-t 16 h postinfection). Coinfection f this virus with the Ad EII-E-CAT virus did not cause transactivation of the EII-early promoter at 10 h after infection (45a).

As mentioned above, small-t was capable of transactivating the Ad EIII promoter in transient transfection assays. To determine whether Ad-SV-t would transactivate this RNA polymerase II promoter, we assayed transcription of the EIII promoter in single-infection experiments. CV-1 or HeLa cells were infected with *dl*309 (EIA$^+$), *dl*321 (EIA$^-$), or Ad-SV-t at 25 PFU per cell for 8 or 16 h, and transcription from the EIII gene of the same virus was quantitated by the extension of labeled primers complementary to the EIII mRNAs of the virus. The presence of EIA in the virus elicited an approximately 10-fold increase in the transcription of the EIII gene compared with EIII gene expression in the absence of EIA at 8 h postinfection (Fig. 6, lanes 1 and 2). However, we did not see a comparative increase in the EIII transcription of Ad-SV-t (lane 3). At 16 h postinfection, there was no increase in EIII transcription in our recombinant virus compared with the EIA-negative *dl*321 (lanes 4 to 9). As has been reported by others, we have observed decreased transcription of the EIII promoter at late times (4). One possible explanation for this finding is that the ara-C used to block DNA replication in these experiments did not function at maximum efficiency.

We considered the possibility that our failure to observe transactivation of polymerase II promoters in virus infections resulted from an inhibitory effect of small-t on the transactivation mechanism when the protein was expressed by an enhancer/promoter in the context of the viral chromosome; for instance, there might be competition for a limiting transcription factor. To rule out any negative effects f the small-t expression system on the transactivation of RNA polymerase II promoters, we studied the EIA-mediated transactivation of the EII-early promoter in the presence f small-t. CV-1 cells were coinfected either with *dl*309 (EIA$^+$) and the EII-early CAT vector or with *dl*309 (EIA$^+$), Ad-SV-t, and the EII-early CAT virus for 16 h; all infections were carried out at 25 PFU per cell. Comparable transactivation of the EII-early promoter was observed both in the presence and in the absence of the small-t antigen (Fig. 7).

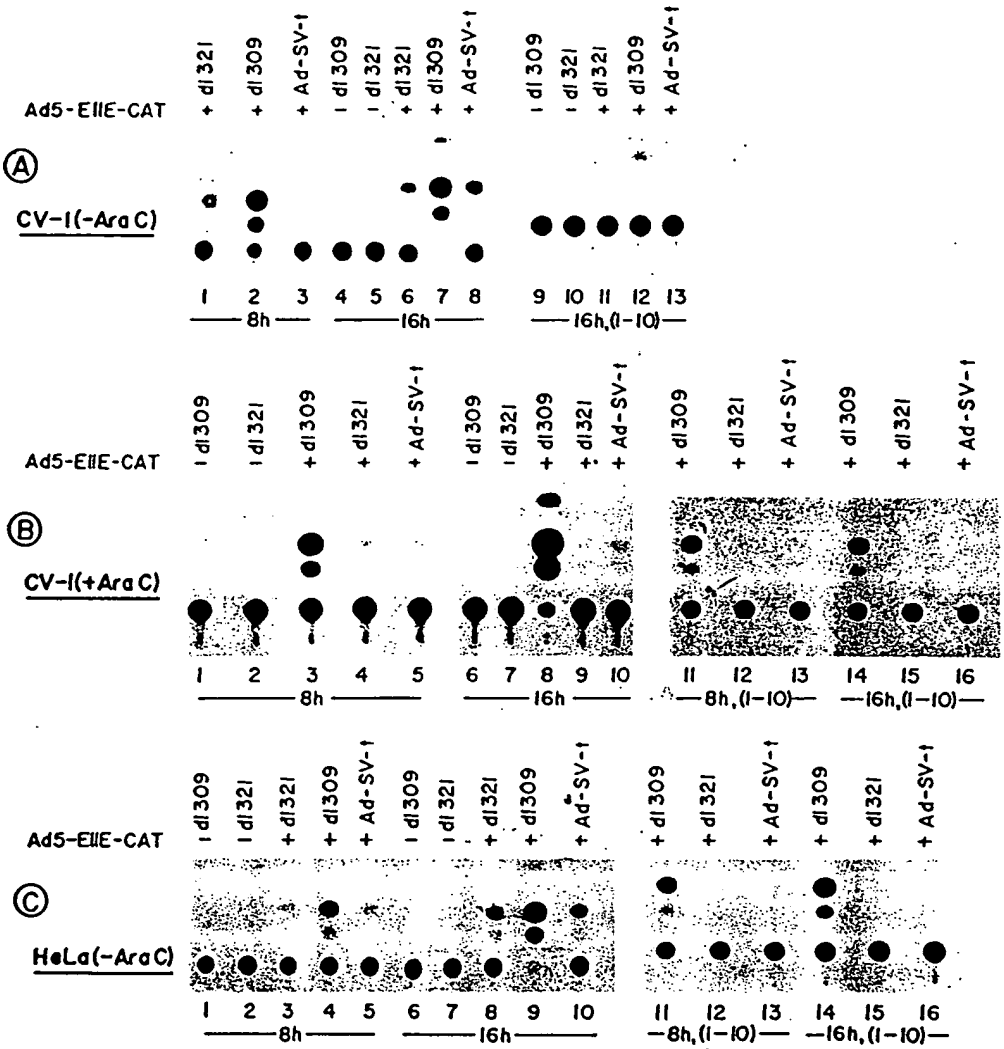

FIG. 5. Transcriptional stimulation of the EII-early promoter by EIA or small-t in virus infections. (A) Transactivation of the Ad EII-early promoter by EIA or small-t in CV-1 cells in the absence of ara-C. The cells (60-mm dishes) were infected with *dl*309 (EIA$^+$), *dl*321 (EIA$^-$), and Ad-SV-t in the absence or presence of Ad EII-E-CAT as indicated. All infections were carried out at 25 PFU per cell. Cells were harvested at the indicated time points, and 50 μg of the protein was used for CAT assays (18). Lanes 9 to 13 represent CAT activity for 16-h samples after 10-fold dilution. (B) Transactivation of the Ad EII-early promoter by EIA or the small-t in CV-1 cells in the presence of ara-C. Infections were carried out in the presence of 25 μg of ara-C per ml. Other details are as for panel A. Samples in lanes 3 to 5 were diluted 10-fold and assayed in lanes 11 to 13, and samples in lanes 8 to 10 were diluted 10-fold and assayed in lanes 14 to 16. (C) Transactivation of the Ad EII-early promoter by EIA or the small-t in HeLa cells in the absence of ara-C. Experimental details are as for panel A. Samples in lanes 3 to 5 were diluted 10-fold and assayed in lanes 11 to 13, whereas samples in lanes 8 to 10 were diluted 10-fold and assayed in lanes 14 to 16.

Ad-SV-t-encoded small-t can transactivate the EII-early promoter when the promoter is introduced into cells by transient transfection. We then considered the possibility that transactivation of the EII-early promoter by small-t is dependent on the state of the target gene. If this is true, the Ad-SV-t-encoded small-t would transactivate the EII-early promoter when CV-1 cells are infected with Ad-SV-t and transfected with EII-early CAT plasmid. CV-1 cells were transfected with EII-early CAT for 60 h and then infected with Ad-SV-t at 25 PFU per cell in the presence of ara-C. Sixteen hours following infection, cells were lysed and CAT activity was determined (18). Mutant *dl*309 (EIA$^+$) infection was also carried out as a positive control. After correction for transfection efficiency, we found that both EIA and small-t stimulated EII-early promoter by approximately five-fold (Fig. 8 and Table 1). Thus, it seems likely that one of the requirements for small-t-mediated transactivation is that the target gene be present in the cell in a transiently transfected form.

DISCUSSION

Although the SV40 small-t antigen plays an auxiliary role in transformation, particularly when growth-arrested cells are used, a role for this protein in the virus growth cycle is yet to be established. It may be mentioned, however, that small-t mutants of SV40 were found to replicate with a lower efficiency in some cell lines than did its wild-type counterpart (42). The small-t antigen is required for the anchorage-independent growth of SV40-transformed cells at limiting

TABLE 1. Effect of small-t on EII-early promoter expression in transient transfections and virus infections in CV-1 cells[a]

Expt	Route of introduction of activator or promoter	Product formed (nmol)	Fold induction
1	Activator and reporter by transient assays		
	EII-early promoter (pEII-E-CAT)		
	Control (pGEM)	0.02	1.0
	+E1A (pCD2)	0.56	29.4
	+Small-t (pW2t)	0.59	30.9
	EIII promoter (pEIII-CAT)		
	Control (pGEM)	0.015	1.0
	+EIA (pCD2)	0.52	35.7
	+Small-t (pW2t)	0.15	10.3
2	Activator and reporter by virus infections		
	EII-early promoter (Ad-EII-E-CAT), 16 h		
	Control (*dl*321)	0.92	1.0
	+EIA (*dl*309)	10.3	11.3
	+Small-t (Ad-SV-t)	1.05	1.14
	EII-early promoter, 24 h		
	Control (*dl*321)	5.0	1.0
	+EIA (*dl*309)	7.9	1.58
	+Small-t (Ad-SV-t)	7.4	1.48
	EII-early promoter, 16 h (+araC)		
	Control (*dl*321)	0.004	1.0
	+EIA (*dl*309)	0.54	154
	+Small-t (Ad-SV-t)	0.005	1.4
	EII-early promoter, 24 h (+araC)		
	Control (*dl*321)	0.005	1.0
	+EIA (*dl*309)	0.49	91
	+Small-t (Ad-SV-t)	0.006	1.1
3	Activator by virus infection, reporter by transfection		
	EII-early promoter (pEII-E-CAT)		
	Control (*dl*321)	0.17	1.0
	+EIA (*dl*309)	0.84	4.9
	+Small-t (Ad-SV-t)	0.92	5.4

[a] Data for experiments 1, 2, and 3 are quantitated from results obtained in Figs. 1, 5, and 8, respectively. See Materials and Methods and figure legends for details. For experiments 1 and 3, CAT assays were performed by incubating the lysates for 2 h rather than the standard incubation time (30 min). Assays were always performed under conditions in which the conversion of [^{14}C]chloramphenicol to its products was less than 30% of the total substrate added. Samples were diluted whenever necessary to obtain values within this range.

concentrations of large-T (5). Also, the small-t protein has been reported to disrupt intracellular actin cables in some instances of nonpermissive infection (19), although contradictory results have been obtained (35). In SV40-infected monkey cells, small-t has been found to be associated with two cellular proteins of molecular sizes 61 and 37 kDa (37). Recent studies show that these proteins correspond to the A and C subunits, respectively, of the cellular enzyme serine- and threonine-specific protein phosphatase 2A (PP2A) (34, 50). The observation that small-t could transactivate the Ad EII-early promoter and VA gene expression to levels comparable to those produced by the better-documented Ad EIA protein in transient transfection studies (29) opened up a field of new possibilities. We have used four independent approaches to further study this observation. In the first approach, transactivation of the polymerase II promoters was studied in constructed CV-1 cell lines which constitutively express small-t. The EII-early promoter was introduced into these cells by infection or transfection. In the second approach, both the activator and the target genes were introduced into cells by transient transfection. In the last two approaches, small-t was introduced into cells by virus infection, whereas the target genes were present either in the context of the viral chromosome or in a transient transfected state. Efficient stimulation of polymerase II pr moters was observed only when the reporter gene was introduced into the cells by transient transfection. The observation that CV-1 cells which constitutively express small-t failed to transactivate the EII-early promoter even when the EII-early promoter was present in the cell in the transfected state is intriguing and is not consistent with this conclusion. Perhaps these cells are desensitized for the small-t effect (see below). Notwithstanding this observation, our data suggest that transactivation of polymerase II promoters by small-t occurs reproducibly in transient assays and thus may depend on the state of the target gene. This is in striking contrast to the E1A-mediated transactivation effect which can be observed regardless of the context in which the target gene is expressed.

Because data accumulated until now indicate that small-t is not a DNA-binding protein (36), small-t probably does not carry out its proposed transactivation function directly. The association of small-t with PP2A may have a role in the small-t-mediated transactivation function. The lack of transactivation by small-t in small-t-expressing cell lines could be explained by the argument that the constant presence of the small-t protein in the cell has by some means desensitized the cell to the effects of small-t. For example, cell lines may adapt to the chronic presence of small-t by slightly increasing PP2A levels such that PP2A is in excess over small-t. Modest increases would be quite difficult to detect using single cell lines in which clonal variability could also play a role.

Why does small-t transactivate polymerase II pr moters in transfection assays but not in a virus infection? It seems unlikely that the small-t expressed in Ad-infected cells is

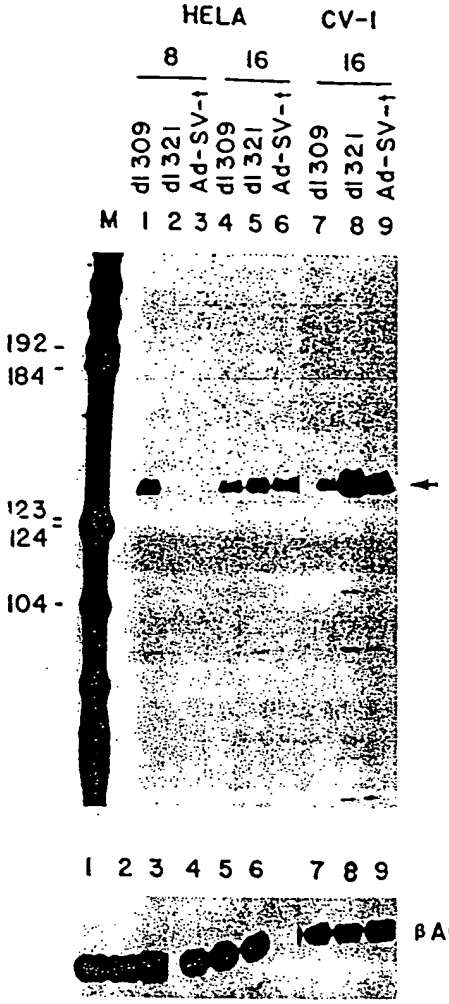

FIG. 6. Transcriptional transactivation of the Ad EIII promoter by EIA or small-t in CV-1 and HeLa cells. CV-1 or HeLa cells were infected with *dl*309 (EIA$^+$), *dl*321 (EIA$^-$), or Ad-SV-t at 25 PFU per cell for 8 or 16 h in the presence of 25 μg of ara-C per ml. Protocols for the extraction of total cytoplasmic RNA and the primer extension reaction are described in Materials and Methods. A 5'-end-labeled *Hae*III digest of pBR322 DNA was used as markers (lane M; indicated in nucleotides). The extension product of the EIII primer is 133 nucleotides and is denoted by the arrow. Quantitational normalization of the RNA in each of the lanes was done with β-actin mRNA. The product of this primer extension reaction is shown at the bottom.

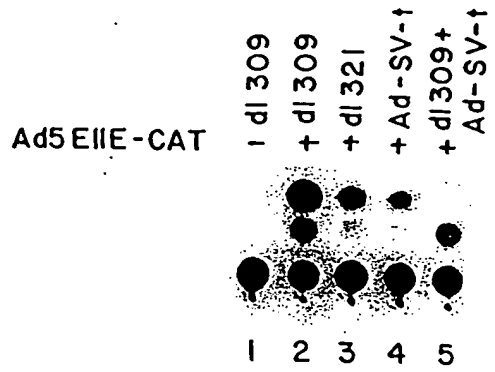

FIG. 7. Activation of the EII-early promoter by EIA in the presence of the small-t protein. CV-1 cells were coinfected with viruses indicated. All infections were carried out for 16 h at 25 PFU per cell in the presence of 25 μg of ara-C per ml. Cell lysates were then prepared, and CAT activity was estimated.

biologically inactive, as our results show that small-t expressed by Ad-SV-t can transactivate the EII-early promoter when the promoter is present in transfected state. In addition, there are several examples in which Ad vectors were used to express biologically functional proteins. The SV40 large-T antigen expressed from an Ad vector retains its DNA replication functions (47), and the polyomavirus middle-T antigen also retains its biological functions when expressed from an Ad vector (3, 12, 39). Because basal and EIA-induced expression of the CAT gene from the Ad EII-early promoter was not hampered by the presence f small-t (Fig. 5), it is unlikely that the enhancer/promoter expression system for small-t in our recombinant viruses, Ad-SV-t and Ad-CMV-t (45a), is competing for a limiting transcription factor. It is possible that in transient transfection assays, the chromatin structure of the target gene is considerably different from that of the viral or host chromosome, which may be sensitive to the small-t effect. Alternatively, there may be a compartmentalization effect when transfected and infected genes enter different locations in the nuclei where they have varied access to different transcription factors. Recent results from our laboratory confirm the fact that discrepancies do seem to arise between experiments that involve infection and transfection protocols (30). In a detailed study of the transcriptional regulation of the Ad EII-early promoter, we previously showed that in transfection assays, promoter mutants with mutations in the TAGA, ATF, or two EIIF motifs were all induced by EIA (31). However, when these mutants were reinserted into the virus genome and therefore analyzed in the context of the viral chromosome, none of the mutants were induced by EIA, indicating that the four

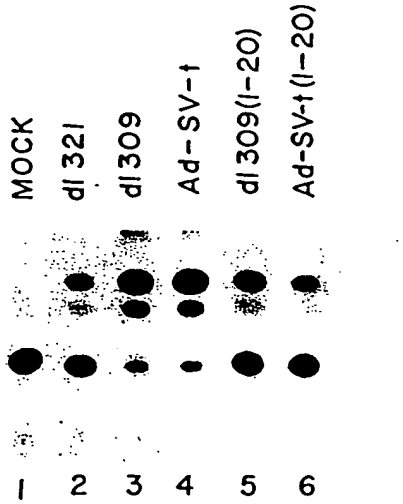

FIG. 8. Transactivation of the EII-early promoter in CV-1 cells when cells were transfected with the EII-early CAT plasmid and infected with Ad-SV-t or *dl*309 (EIA$^+$). CV-1 cells (30-mm dishes) were first transfected with 2 μg of the EII-early CAT plasmid for 60 h and then infected with different Ad mutants at 25 PFU per cell in the presence of 40 μg of ara-C per ml. Cells were lysed 16 h after infection, and equal amounts protein were used for CAT assays. Samples in lanes 3 and 4 were diluted 20-fold and reassayed in lanes 5 and 6, respectively.

upstream elements, i.e., the ATF, TAGA, and the two EIIF sites, acted in concert to regulate the EIA induction of the promoter, contradictory to the conclusions drawn from transfection data (30). The same data are also indicative of the fact that transfection assays do not accurately mimic the ongoing regulation of virus-infected cells. A number of viral genes have been shown to possess transactivation properties in transient transfection assays. It will be important to examine whether the transactivation property f these genes is confined to transfection assays as is reported here, or whether their induction capacities extend to several assay systems as does the Ad EIA.

ACKNOWLEDGMENTS

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Adenovirus E1A under the Control of Heterologous Promoters: Wide Variation in E1A Expression Levels Has Little Effect on Virus Replication

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Adenovirus early region 1A (E1A) encodes a heterogeneous family of proteins some of which function as transactivators and are required for efficient viral replication in HeLa cells. We have constructed adenovirus type 5 (Ad 5) mutants in which the E1A transcription unit is placed under the control of either the human β-actin promoter or the human cytomegalovirus immediate early promoter. The level of E1A expression in cells infected with these mutants was several times higher than that in wild-type virus infections. When the same heterologous promoters were inserted upstream of, but in the opposite orientation to, the E1A transcription unit, the level of expression was greatly reduced with respect to wild-type levels of E1A. Despite this variation of at least 40-fold in the concentration of E1A proteins in infected cells, there was no significant difference between wild-type Ad 5 and any of the mutants in their ability to replicate in HeLa cells. These results suggest that very low levels of E1A proteins are sufficient for virus production in cultured cells and that wild-type Ad 5 produces an amount of E1A in excess of that required.

INTRODUCTION

Early region 1A (E1A) of adenovirus is the first region of the viral genome to be expressed in infected cells (Nevins *et al.*, 1979). The primary E1A transcript is differentially spliced to produce at least five mRNAs, four of which (13 S, 12 S, 11 S, and 10 S) differ in size and number of introns, but maintain the same reading frames and consequently encode highly related proteins (Perricaudet *et al.*, 1979; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987). The fifth message, a 9 S transcript, shares the same 5′ and 3′ ends with the other E1A mRNAs, but is spliced such that its second exon is predicted to be translated in a different reading frame (Virtanen and Pettersson, 1983). The 12 S and 13 S mRNAs are expressed both early and late after infection (Berk and Sharp, 1978; Chow *et al.*, 1979), whereas the 9 S, 10 S, and 11 S transcripts are detected only at late times (Ulfendahl *et al.*, 1987). All of the E1A gene products are post-translationally modified, principally by phosphorylation (Gaynor *et al.*, 1982; Yee *et al.*, 1983), giving rise to many different species of E1A proteins which can be distinguished by their electrophoretic mobility (Yee and Branton, 1985a; Harlow *et al.*, 1985).

E1A plays a role in a number of important biological functions in adenovirus-infected cells. E1A proteins are capable of immortalizing primary cells and of cooperating with E1B or H-*ras* to induce complete transformation (reviewed in Graham, 1984) and are also involved in regulation of gene expression. Transcriptional activation of E1A and other early viral promoters, as well as of a number of cellular promoters, can be induced by E1A gene products (reviewed in Berk, 1986). In addition, both cellular (Hen *et al.*, 1985; Stein and Ziff, 1987; Webster *et al.*, 1988) and viral (Borrelli *et al.*, 1984) enhancers, including the E1A enhancer itself, can be repressed by E1A proteins. Repression and activation are unlikely to be direct effects of E1A on enhancers and promoters since E1A does not bind DNA in a sequence-specific manner (Ferguson *et al.*, 1985; Chatterjee *et al.*, 1988). However, several cellular proteins are known to bind E1A products (Yee and Branton, 1985b; Harlow *et al.*, 1986); thus E1A might regulate expression by interacting with cellular mediators (Berk, 1986).

The ability of E1A to both activate and repress its own transcription complicates the analysis of E1A mutants. Mutations in E1A which affect autoregulation might indirectly affect other functions through changes in the level of E1A protein synthesis. As a preliminary attempt to overcome this problem, we have constructed viruses in which expression of the E1A gene has been placed under the control of heterologous promoters. An additional motive for constructing such viruses was to enable us to correlate the sensitivity of various E1A functions, such as transformation and virus replication, to changes in the levels of E1A expression.

Two promoters were chosen as most suitable for this study. The β-actin promoter has been shown to function at high levels in transient expression assays in

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a wide range of cell types (Gunning *et al.*, 1987; Sugiyama *et al.*, 1988) and appears to be insensitive to E1A products (Webster *et al.*, 1988). In addition, we have utilized the human cytomegalovirus (HCMV) immediate early (IE) enhancer/promoter which was shown to induce very high levels of expression and to have little cell type or species preference (Boshart *et al.*, 1985). It is not clear, however, whether or to what degree the HCMV IE promoter is sensitive to adenovirus E1A proteins.

We have constructed mutant adenoviruses by inserting each of these two promoters between the wild-type E1A transcription initiation site and the E1A open reading frame. The analysis of both E1A expression and E1A function in cells infected with the mutant viruses is described here.

MATERIALS AND METHODS

Construction of recombinant plasmids

All enzymes used in cloning were purchased from Bethesda Research Laboratories (Burlington, Ontario), New England BioLabs (Beverly, MA), or Boehringer-Mannheim, Inc. (Dorval, Quebec). Plasmids were constructed by standard methods (Maniatis *et al.*, 1982) and used to transform (Mandel and Higa, 1970) competent bacterial cells (LE392 except as noted). Plasmid DNA was prepared by the alkaline lysis method of Birnboim and Doly (1979).

Plasmid pX548c was constructed by D. Bautista *et al.* (manuscript in preparation). This construct (derived from pXC1; McKinnon *et al.*, 1982) contains all of the Ad 5 *Xho*I C fragment with a unique *Bam*HI restriction site at nucleotide 548 of Ad 5 generated by linker insertion (Fig. 1A).

The human β-actin promoter was obtained originally from the plasmid pHβAPr-1 (a gift of D. Huszar, Whitehead Institute, MA) described by Gunning *et al.* (1987). The *Sac*I fragment of pHβAPr-1 (from approximately −2000 to −450 relative to the β-actin transcription start site) was deleted and replaced with a *Bam*HI linker to generate plasmid pβAPrΔSac. Subsequent *Bam*HI digestion released a 1.4-kb DNA fragment carrying the β-actin promoter flanked by linker sequences. This fragment was ligated to *Bam*HI-digested pX548c, and plasmids were selected which contained the β-actin promoter in the same (pβ548-1) or opposite (pβ548-2) orientation as the E1A transcription unit.

The HCMV immediate early promoter was isolated from the plasmid pUCIEPenv (a gift of G. W. Wilkinson, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury) in which the IE promoter is flanked by an *Eco*RI site upstream and a *Bam*HI site downstream (G. W. Wilkinson, personal communication). pUCIEPenv was digested with *Eco*RI and made blunt ended with the Klenow fragment of DNA polymerase I. *Bgl*II linkers were ligated to the fragment which was subsequently digested with *Bgl*II and *Bam*HI. The 0.38-kb fragment containing the promoter was inserted at position 548 of pX548c. Plasmids were selected which contained the HCMV IE promoter inserted in the same (pIEP548-1) or the opposite (pIEP548-2) orientation as the E1A transcription unit.

In order to construct a reverse orientation promoter-insertion mutant unable to synthesize the 13 S E1A gene product, a plasmid was generated containing a stop codon within the region of E1A unique to the 13 S transcript. The first step in generating this plasmid was to construct pIEP2-ΔStu by *Stu*I digestion of pIEP548-2 (grown in GM48 to prevent methylation of the *Stu*I recognition sequence) and religation. This removed the Ad 5 sequence between nucleotides 3157 and 5779, which contains two *Xma*I sites (at 3940 and 4120), and facilitated further manipulations (below). Insertion of the synthetic adaptor

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5'-CCGGTTAAGCTTTAA
      AATTCGAAATTGGCC-5'
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at the *Xma*I site (nucleotide 1007) of pIEP2-ΔStu resulted in both the disruption of the *Xma*I site and the introduction of a stop codon and a diagnostic *Hin*dIII site, generating plasmid pIEP2-1008.

Cells and viruses

All tissue culture reagents were purchased from GIBCO Laboratories (Grand Island, NY). All growth media were supplemented with 2 m*M* glutamine and with antibiotics (100 units penicillin G/ml and 100 μg streptomycin sulfate/ml). HeLa cells were grown in monolayer cultures in α-minimum essential medium supplemented with 10% fetal calf serum (complete αMEM). The human E1-expressing 293 cell line (Graham *et al.*, 1977) was grown in monolayer culture in F11 medium supplemented with 10% newborn calf serum.

Wild-type and dl312 (Jones and Schenk, 1979) Ad 5 viruses were propagated and titrated on 293 cells. Plasmids containing mutated E1A genes were rescued into infectious adenovirus as described by McGrory *et al.* (1988). Briefly, 293 cells were cotransfected by the calcium phosphate method (Graham and Van der Eb, 1973) with mutant plasmid DNAs and pJM17 (a circular form of Ad 5 which replicates in bacterial cells due to insertion of pBR322 DNA into the E1A region). Most (50 to 100%) of the plaques obtained after cotransfection were recombinants, due to the size constraints which prevent packaging of the 40.3-kb pJM17-derived

viral progeny. Recombinant viruses were selected and plaque-purified three times before being grown in large scale on 293 cells.

Virus growth curves were obtained by infecting subconfluent monolayers of HeLa cells with wild-type or recombinant virus at an m.o.i. of 10 PFU per cell. At various times postinfection the cells were harvested by scraping and combined with the spent medium. This crude virus stock was frozen and thawed three times before titrating by plaque assay on 293 cells.

Primer extension analysis

Total cellular RNA was isolated by the guanidinium–CsCl method (Ullrich *et al.*, 1977) from 150-mm dishes of HeLa cells 12 hr after infection with wild-type or recombinant virus at an m.o.i. of 30 PFU per cell. Primer extension experiments used an end-labeled synthetic oligonucleotide primer (5′-γTATCAGCCAGTACCTCT-3′) purchased from the Central Facility of the Institute of Molecular Biology and Biotechnology, McMaster University. The primer (0.6 pmol), which hybridizes to nucleotides 634 to 650 of Ad 5, was annealed to 40 μg of RNA and extended with AMV reverse transcriptase (Life Sciences, St. Petersburg, FL) as described by Smiley *et al.* (1987). The extension products, as well as size markers prepared by end-labeling a mixture of *Hin*fI- and *Hin*cII-digested pBR322, were separated by electrophoresis through a 4.5% polyacrylamide sequencing gel for 1.5 hr at 2000 V. The gel was then dried and exposed to Kodak X-Omat AR film at −70°.

Radioactive labeling of proteins

Prior to [^{35}S]methionine labeling for E1A detection, HeLa cells were grown to approximately 70% confluence in 60-mm dishes, then infected at an m.o.i. of 30 PFU per cell with either wild-type Ad 5 or one of the recombinant viruses. After 30 min at room temperature, the cells were overlaid with 5 ml complete αMEM and incubated at 37° for the desired time. Two and one-half hours prior to harvest, the cells were washed with PBS and incubated in 3–5 ml of medium 199 lacking methionine and supplemented with 2% dialyzed fetal calf serum, 1× MEM vitamin solution, 20 m*M* HEPES, pH 7.3, glutamine, and antibiotics (methionine-free medium). After 45 min at 37°, the medium was replaced with 2 ml fresh methionine-free medium supplemented with 30 μCi [^{35}S]methionine (Tran ^{35}S-label, 1000 Ci/mmol, ICN Biomedicals, Inc., Irvine, CA). The cells were labeled for 1.5 hr at 37°, washed once with ice-cold PBS, and harvested by scraping in 0.5 ml 50 m*M* Tris–Cl, pH 7.5, 150 m*M* NaCl, 0.1% sodium dodecylsulfate (SDS), 1% sodium deoxycholate, 1% Triton X-100, 35 μg phenylmethylsulfonyl fluoride/ml, 10 μg aprotinin/ml (RIPA buffer). The extract was vortexed for 5–10 s, then centrifuged 30 min in an Eppendorf microfuge at 4° to pellet debris. To compare expression of E1A to that of E1B and E2, the above protocol was modified as follows: 100-mm dishes of HeLa cells were infected for various times, labeled with 150 μCi [^{35}S]methionine, and harvested in 1.5 ml RIPA buffer. Each extract was divided into three portions for immunoprecipitation of the early gene products.

For ^{32}P-labeling, 100-mm dishes of HeLa cells were infected as above, and after 15.5 hr at 37°, the cells were washed once with medium 199 lacking phosphate, supplemented as described for methionine-free medium. The cells were labeled in 2 ml phosphate-free medium supplemented with approximately 0.5 mCi [^{32}P]orthophosphate (HCl-free, carrier-free, ICN Biomedicals) for 2.5 hr, then harvested in 1 ml RIPA buffer.

Immunoprecipitation and electrophoresis

E1A-specific proteins were immunoprecipitated from HeLa extracts by mixing 0.5 ml labeled cell extract with 1 μg of the monoclonal antibody M73 (Oncogene Science, Inc., NY; Harlow *et al.*, 1985) and 100 μl of a 50% slurry of protein A–Sepharose CL-4B (Pharmacia Canada, Inc., Quebec) in RIPA buffer for 2 hr at 4°. The Sepharose was then washed extensively in RIPA buffer and resuspended in 50 μl 2× loading buffer (80 m*M* Tris–Cl, pH 6.8, 2.5% SDS, 25% glycerol, 1 *M* β-mercaptoethanol, 0.67 *M* urea, 0.001% bromphenol blue), boiled for 3 min, then centrifuged. Samples were immediately applied to SDS–polyacrylamide gels as detailed below. Other adenovirus early gene products were detected by immunoprecipitation (as described above) with mouse monoclonal antibody (H2-19, gift of S. Bacchetti; Branton *et al.*, 1985) specific for the E2 72-kDa protein or with rabbit antisera raised against peptides corresponding to the C-terminus of either the 58-kDa or the 19-kDa E1B product (58C-2 or 19C-24A, respectively, gifts of P. E. Branton).

^{35}S-labeled supernatants were electrophoresed at 5 mA overnight through 0.1% SDS–10% polyacrylamide gels as described by Laemmli (1970). ^{32}P-labeled samples were fractionated on 12% polyacrylamide gels. ^{14}C-methylated proteins (Amersham Canada, Ltd., Ontario) were used as markers for ^{35}S-labeled protein gels, and prestained protein molecular weight standards (Bethesda Research Laboratories) were used to calibrate ^{32}P-labeled protein gels. ^{35}S-labeled proteins were detected by fluorography (Bonner, 1984) on unflashed Kodak X-Omat AR film and quantitated by densitometry. ^{32}P-labeled proteins were detected by autoradiography and quantitated by cutting out spots on the dried gel corresponding to E1A-specific bands, di-

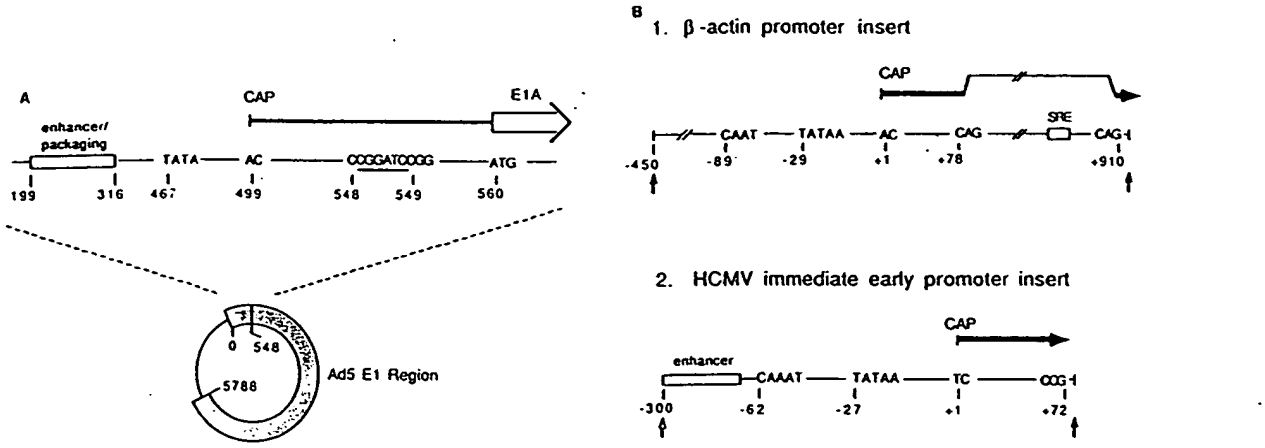

FIG. 1. Structures of Ad 5 and heterologous promoter plasmids. (A) The Ad 5 *Xho*I C fragment (shaded region) carried by plasmid pX548c is depicted below, with an expanded version of the sequence near the E1A promoter shown above (not to scale). The Ad 5 sequence in pX548c differs from wild-type only by insertion of a *Bam*HI linker (underlined) at nt 548. DNA is numbered relative to the left end of wild-type Ad 5. The bold horizontal arrow corresponds to the sequence and direction of transcription, and the large open arrow denotes the E1A open reading frame. The position of the enhancer is indicated by an open box. (B) The structures of the heterologous promoters inserted at the *Bam*HI site of pX548c are shown. Nucleotides are numbered with respect to the normal transcription initiation site of the given gene. The narrow horizontal line indicates an intervening sequence in the mature β-actin transcript and the serum responsive element of β-actin is indicated SRE. Closed vertical arrows indicate *Bam*HI sites, the open vertical arrow indicates a *Bgl*II site, and all other symbols are as described in (A) above. Mutants containing the β-actin or HCMV IE promoter in the same orientation as the E1A transcription unit are designated β548-1 and IEP548-1, respectively. Mutants containing promoters inserted in the opposite orientation are designated β548-2 and IEP548-2.

gesting them overnight at 37° in 95 parts 30% H_2O_2–5 parts concentrated NH_4OH, then counting in 10 volumes aqueous scintillation fluid.

RESULTS

Structure of recombinant plasmids and viruses

In order to construct viruses capable of expressing altered levels of E1A protein, two sets of E1A mutants were generated which contained heterologous promoters inserted 5′ to the E1A open reading frame. The E1A enhancer and promoter were not deleted in these mutants because the sequences required for virus packaging overlap with the enhancer (Hearing and Shenk, 1986), and our objective was to rescue the mutations into intact virus. To generate our mutants, we utilized the plasmid pX548c, containing a *Bam*HI site between the transcription initiation site and the E1A coding sequence (Fig. 1A). One set of mutants was constructed by inserting a 1.4-kb DNA fragment containing the human β-actin promoter. This fragment of the β-actin gene (from approximately position −450 to +910 relative to the transcription start site) includes the upstream CAAT and TATA box elements, a serum responsive enhancer element, and intervening sequence one, terminating at the splice acceptor site so that the normal translation start site (+917) is absent (Ng *et al.*, 1985, 1989) (Fig. 1B). Plasmids were selected which carried the β-actin promoter either in the same orientation as the E1A open reading frame (pβ548-1) or in the opposite orientation (pβ548-2).

The second set of mutants contained the HCMV IE promoter sequence from −299 to +72 inserted at the *Bam*HI site of pX548c. This fragment of the promoter includes part of the enhancer, the CAAT and TATA box elements, the transcription initiation site, and 72 bp corresponding to the beginning of the 5′ untranslated sequence of the 1.9-kb IE transcript (Boshart *et al.*, 1985) (Fig. 1B). Again, plasmids were constructed with the IE promoter either in the same (pIEP548-1) or in the opposite (pIEP548-2) orientation as the E1A transcription unit.

These mutations in the E1A promoter region were rescued into infectious adenovirus using the cotransfection method developed by McGrory *et al.* (1988). The resulting E1A promoter-insertion viruses (β548-1, β548-2, IEP548-1, and IEP548-2) were then analyzed for their ability to express E1A and to replicate in HeLa cells.

Analysis of E1A transcripts

As mentioned, all of the insertion mutants were constructed such that the E1A promoter was still present, but, relative to the heterologous promoter, distal to the E1A coding sequence. To determine which promoter was utilized in infections with recombinant virus, E1A transcripts were analyzed by primer extension (Fig. 2).

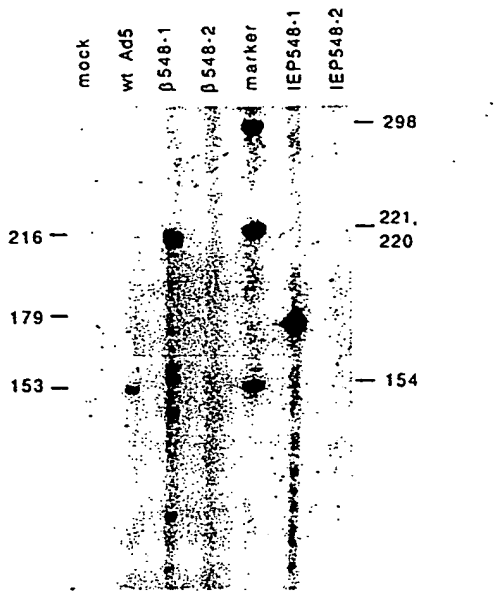

FIG. 2. Analysis of E1A transcription by primer extension. RNA isolated from HeLa cells 12 hr postinfection with wild-type or mutant viruses was annealed to a 5′ end-labeled primer (hybridizing to nts 634 to 650 of Ad 5) and treated with reverse transcriptase as described under Materials and Methods. The cDNA products were fractionated by PAGE. The sizes (in nts) of DNA fragments used as markers are given on the right, and that of the predicted extension products on the left, at positions corresponding to their electrophoretic mobility.

The 5′ end of the primer used in this experiment hybridized to nucleotide (nt) 650 of wild-type Ad 5, generating a 153-nt product in wild-type-infected cells. If the β-actin promoter were used in β548-1 infections, the extended product should be 216 nt in length (78 nt of β-actin sequence, 35 nt of polylinker (Gunning *et al.*, 1987), and 103 nt of E1A). The major cDNA detected was the expected size, although smaller cDNAs were also observed. Utilization of the HCMV IE promoter in IEP548-1-infected cells should have generated a 179-nt product, which again was approximately the size of the major cDNA species observed. Both of these recombinant viruses appeared to induce a higher level of E1A transcription than wild-type Ad 5.

RNA isolated from cells infected with the heterologous promoters in reverse orientation to E1A (β548-2 and IEP548-2) produced very low quantities of cDNA of variable size, none greater than 250 nt, and visible only after longer exposures than those shown in Fig. 2. Any transcripts initiated at the wild-type promoter and reading through the inserts would produce fairly large extension products with this primer (about 1.6 kb for β548-1 and β548-2, and about 0.54 kb for IEP548-1 and IEP548-2). No such products were observed with any sample, although conditions of the primer extension reaction may not have favored their generation, or such transcripts could have been generated and subsequently spliced. We conclude from these analyses that the heterologous promoters were driving E1A transcription at elevated levels in cells infected with β548-1 and IEP548-1. With the reverse promoter mutants β548-2 or IEP548-2 on the other hand, E1A either was not transcribed or was transcribed at very low levels relative to wild-type Ad 5.

Expression of E1A proteins

E1A is normally expressed early after adenovirus infection and maintained at a reasonably high level during the late phase (Nevins *et al.*, 1979). To establish whether E1A expression driven by the β-actin or HCMV IE promoter followed the same kinetics as that under control of the wild-type promoter, E1A protein synthesis in cells infected with recombinant viruses was determined as a function of time postinfection. In the first set of experiments, extracts were prepared from HeLa cells labeled with [^{35}S]methionine at various times after infection with wild-type, IEP548-1, or IEP548-2. E1A proteins were immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described under Materials and Methods. The 7-day exposures of the gels are shown in Fig. 3A. The time course of E1A expression in IEP548-1 infections paralleled that in wild-type infections, but levels of E1A protein were approximately threefold higher in mutant virus infections (Fig. 3B). No E1A products were detected in extracts from cells infected with IEP548-2 until about 18–24 hr postinfection, at which time very low levels were produced. Similar results were obtained when infections with recombinant viruses containing the β-actin promoter were examined (a 3-day exposure of the gel is shown in Fig. 3A; the wild-type infections from this experiment are not shown, but are qualitatively similar to those of the previous experiment). β548-1 induced three times the level of E1A proteins when compared to wild-type, and no E1A protein was detected in β548-2 infections (Fig. 3C). These experiments were repeated several times and in each case, cells infected with the forward promoter mutants expressed elevated levels of E1A protein with kinetics similar to that of cells infected with wild-type Ad 5. E1A protein was never detected in infections with β548-2 and was detectable only in trace amounts with IEP548-2, at late times and only in some experiments.

Analysis of replication of mutant viruses

Since infections with β548-1 and IEP548-1 viruses resulted in overproduction of E1A proteins, and those with β548-2 and IEP548-2 viruses resulted in little (HCMV IE) or no (β-actin) E1A protein synthesis, it was

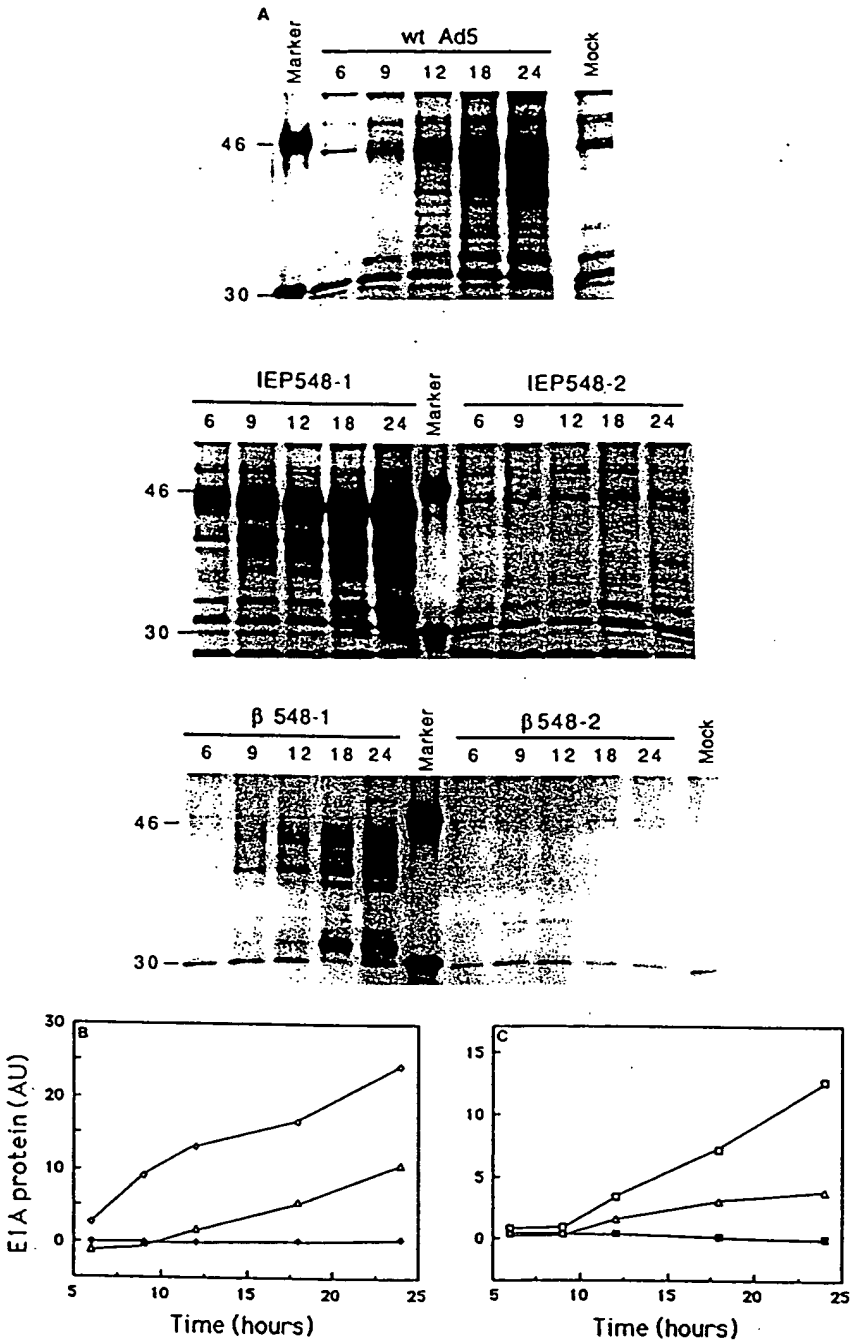

FIG. 3. Time course of E1A expression in infected HeLa cells. (A) At various times postinfection, HeLa cells were labeled with [^{35}S]methionine and harvested as described under Materials and Methods. Extracts were immunoprecipitated with M73 antibody and the resulting E1A proteins separated by SDS–PAGE. Molecular weights of markers are given in kilodaltons at the left. The times at which cells were harvested, in hours postinfection, are given above each autoradiograph. The time course of E1A expression in cells infected with viruses containing the HCMV IE promoter was determined in a separate experiment (7-day exposures of the gels are shown) from that in cells infected with viruses containing the β-actin promoter (a 3-day exposure of the gel is shown). Only the expression of E1A in wild-type-infected cells from the former experiment is given. (B, C) Approximate amounts of E1A proteins produced during the time course were determined by densitometric scanning of the autoradiographs shown in (A) (and also that from the wild-type infections carried out concurrently with the β-actin promoter mutant infections) and subtracting the background absorbance detected in immunoprecipitations of mock-infected cell extracts. AU, absorbance units. (B) Absorbance of E1A-specific bands in the autoradiograph produced by exposing the film for 7 days to immunoprecipitated proteins from wild-type Ad 5- (Δ), IEP548-1- (◇), and IEP548-2- (♦) infected cell extracts. (C) Absorbance of E1A-specific bands in the autoradiograph after a 3-day exposure to immunoprecipitates from wild-type Ad 5- (Δ), β548-1- (□), and β548-2- (■) infected cell extracts.

TABLE 1

PLAQUE TITRATION OF WILD-TYPE AND RECOMBINANT VIRUS

Virus	Infectivity (PFU/ml) 293	HeLa	HeLa/293
Expt 1			
Wild-type	3.6×10^9	6.1×10^9	1.7
β548-1	6.0×10^8	7.1×10^8	1.2
β548-2	1.7×10^9	2.2×10^8	0.13
IEP548-1	2.1×10^9	1.3×10^9	0.62
IEP548-2	2.4×10^9	1.7×10^9	0.71
Expt 2			
β548-1	1.3×10^{10}	1.2×10^{10}	0.92
β548-2	1.2×10^{10}	1.0×10^9	0.083
IEP548-1	5.0×10^9	4.4×10^9	0.88
IEP548-2	1.5×10^{10}	4.0×10^9	0.27
Expt 3			
Wild-type	9.0×10^9	9.1×10^9	1.0
β548-1	5.0×10^8	3.0×10^8	0.6
β548-2	2.0×10^9	9.0×10^7	0.045
IEP548-1	6.0×10^7	1.2×10^7	0.2
IEP548-2	2.0×10^9	4.3×10^8	0.21
Expt 4			
Wild-type	4.4×10^9	2.6×10^9	0.59
dl312	1.2×10^{10}	6.0×10^5	5.0×10^{-5}
IEP2-1008	3.0×10^9	2.4×10^6	8.0×10^{-4}
Expt 5			
Wild-type	1.7×10^{10}	1.4×10^{10}	0.82
dl312	2.0×10^{10}	2.4×10^6	1.2×10^{-4}
β548-1	1.2×10^9	2.3×10^9	1.9
β548-2	2.9×10^9	1.1×10^9	0.38
IEP548-1	1.4×10^9	2.8×10^9	2.0
IEP548-2	6.3×10^9	6.5×10^9	1.0
IEP2-1008	8.5×10^9	7.6×10^6	8.9×10^{-4}

of interest to examine the relative ability of these viruses to replicate in 293 cells and HeLa cells. Initially replication of the mutant viruses was assessed by plaque titration on the two cell types (Table 1). Although there was some variation in the results of four independent experiments, viruses containing the heterologous promoters in the forward orientation (β548-1 and IEP548-1) were usually about as proficient as wild-type Ad 5 for replication in HeLa cells. Surprisingly, IEP548-2, which did not express detectable E1A mRNA by primer extension analysis and only trace amounts of E1A proteins by immunoprecipitation of [^{35}S]methionine-labeled extracts, was nonetheless capable of plaquing on HeLa cell monolayers at or near wild-type levels. Replication of β548-2 in HeLa cells was slightly reduced compared to wild-type, but was 1000-fold greater than replication of dl312, a mutant virus lacking the entire E1A coding sequence (Jones and Shenk, 1979).

The rates of virus production in HeLa cells infected with wild-type or mutant viruses were determined by one-step growth analysis (Fig. 4). In these experiments, both forward and reverse β-actin promoter mutants were indistinguishable from wild-type in their ability to replicate. Replication of the IEP548-1 virus consistently lagged slightly behind wild-type replication at early times postinfection; however, in most experiments the delay was less pronounced than that shown in Fig. 4. The reverse promoter mutant IEP548-2, on the other hand, was equal to wild-type virus in production of viral progeny in infected HeLa cells. The behavior of the β548-2 and IEP548-2 mutants was in marked contrast to results of infection with the dl312 virus, which showed no significant replication in HeLa cells at an input m.o.i. of 10 PFU per cell. Thus we conclude that, despite our inability to detect significant levels of E1A mRNA or proteins, the reverse promoter mutants were as proficient as wild-type virus in ability to replicate in HeLa cells.

To determine whether this result was due to synthesis of E1A sufficient for wild-type levels of transactivation yet insufficient for detection by primer extension analysis or routine immunoprecipitation, an additional adenovirus mutant, IEP2-1008, was constructed (see Materials and Methods). This virus was identical to IEP548-2 except that an oligonucleotide linker containing a stop codon was inserted between nts 1007 and 1008 to truncate any 13 S E1A products which might be synthesized. Such a mutation would have no effect on the 12 S products since that sequence would be

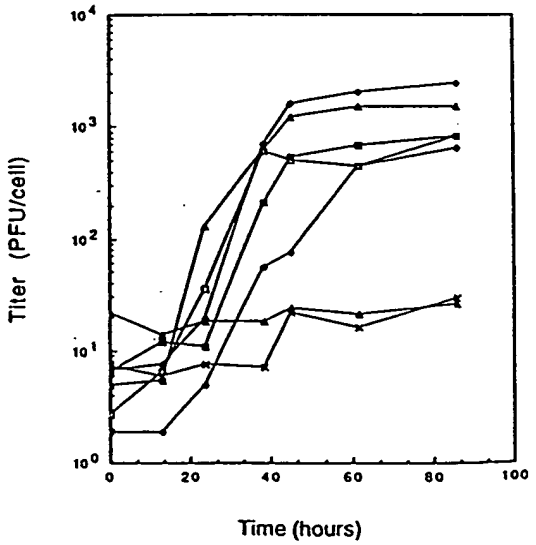

FIG. 4. Wild-type and mutant virus growth curves in HeLa cells. HeLa cells were infected with wild-type or mutant Ad5 viruses, and at various times after infection cells were harvested and titrated on 293 cells as described under Materials and Methods. Wild-type Ad 5 (Δ), dl312 (▲), β548-1 (□), β548-2 (■), IEP548-1 (◇), IEP548-2 (◆), IEP2-1008(×).

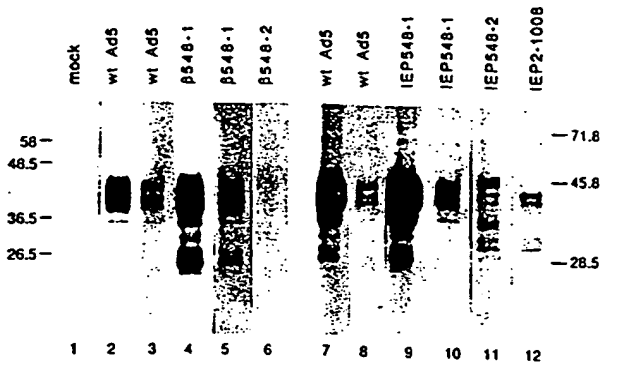

FIG. 5. Immunoprecipitation of ^{32}P-labeled infected HeLa extracts. HeLa cells were infected with wild-type or mutant viruses, labeled with [^{32}P]orthophosphate (600 μCi per dish, for lanes 1–6; or 400 μCi per dish, for lanes 7–12), then harvested. E1A proteins were immunoprecipitated and separated by SDS–PAGE as described under Materials and Methods (lanes 3, 5, 8, and 10 were exposed for 4–5 hr; other lanes were exposed for 18–20 hr). Molecular weight marker sizes (in kilodaltons) are listed to the left and right of the autoradiograph.

spliced out of the 12 S transcript. When the IEP2-1008 virus was tested for replication in HeLa cells, it was found to replicate very poorly (Fig. 4 and Table 1, Experiments 4 and 5), suggesting that replication of the β548-2 and IEP548-2 viruses was due to expression of functional 13 S products, even if at levels undetectable on gels.

Analysis of phosphorylated E1A proteins

In an attempt to increase the sensitivity of E1A protein detection in β548-2 and IEP548-2-infected cells, we examined E1A synthesis by immunoprecipitating ^{32}P-labeled E1A proteins. With this label backgrounds are very low even with long exposures of the gel, presumably due to the limited number of phosphorylated proteins in infected cells which coprecipitate nonspecifically with E1A. Extracts from HeLa cells labeled with [^{32}P]orthophosphate 18 hr after mock infection or infection with wild-type Ad 5, β548-1, or β548-2 were immunoprecipitated and fractionated by SDS–PAGE (Fig. 5, lanes 1–6). Viruses containing the HCMV IE promoters were treated in the same way in a separate experiment (Fig. 5, lanes 7–12). Autoradiography of the gels clearly shows that, as was the case in the [^{35}S]methionine-labeling studies, phosphorylated forms of E1A protein were overproduced in cells infected with β548-1 and IEP548-1. Long exposure of the gels revealed a trace amount of E1A protein produced in β548-2-infected cells. E1A proteins were more readily detected in IEP548-2-infected cells, although at least some of these species seemed to have shifted slightly in mobility with respect to wild-type Ad 5 and the forward promoter mutants (compare lane 11 to lanes 5 and 7 through 10). The IEP2-1008 infection produced at least four proteins (most likely 12 S and 10 S products) which were expressed at about the same level as, and which comigrated with, proteins from IEP548-2 infections (lanes 12 and 11, respectively). Neither the truncated 13 S products from IEP548-2 infection nor the 9S product from any infection would have been precipitated in this experiment, due to the specificity of the M73 antibody for E1A carboxy termini.

Quantitation of phosphorylated E1A species (Table 2) revealed that they were overproduced by 2.4- to 10-fold in IEP548-1 infections, and by 3- to 5-fold in β548-1 infections with respect to wild-type. E1A expression in IEP548-2 infections was reduced about 8-fold with respect to wild-type in Experiment 2. Although the β548-2 mutant apparently produced a very small amount of E1A protein (as detected by visual examination of the autoradiograph), the amount of radioactivity in the E1A bands was not significantly above background. We conclude from these results that, although both the β548-2 and the IEP548-2 reverse promoter mutants were producing functional E1A protein, this was at levels at least 8-fold lower than wild-type Ad 5, and at least 20- to 40-fold lower than the mutants with

TABLE 2

QUANTIFICATION OF ^{32}P-LABELED E1A IMMUNOPRECIPITATED FROM INFECTED HELA EXTRACTS

Virus	E1A protein (cpm)[a]	Percentage of wild-type E1A protein
Expt 1[b]		
Wild-type	2520	(100)
β548-1	7230	290
β548-2	<180	<7
Expt 2		
Wild-type	2600	(100)
IEP548-1	6160	240
IEP548-2	340	13
IEP2-1008	210	8
Expt 3		
Wild-type	511	(100)
β548-1	2700	530
β548-2	<180	<35
IEP548-1	5200	1000
IEP548-2	<180	<35
IEP2-1008	<180	<35

[a] The amount of radioactivity in the lane containing the mock infection sample (350 cpm for Experiment 1, 450 cpm for Experiment 2, and 500 cpm for Experiment 3) was subtracted from the amount of radioactivity in lanes containing the other samples.

[b] Experiments 1 and 2 are described in the legend to Fig. 5. Experiment 3 was performed in exactly the same manner, labeling with 0.5 mCi [^{32}P]orthophosphate per dish.

the corresponding promoters in the forward orientation.

Analysis of other early gene products

Our finding that the reverse orientation promoter mutants β548-2 and IEP548-2 produced very little E1A protein but were capable of replicating in HeLa cells at wild-type levels suggested that, in wild-type infections, E1A proteins are produced in excess of the amounts required for viral replication. It was not apparent whether the low levels of E1A produced in β548-2 and IEP548-2 infections were sufficient to induce wild-type levels of early gene expression and thus high levels of viral replication as well, or if transactivation of other early genes in β548-2 and IEP548-2 infections was reduced but still sufficient for induction of replication. To distinguish between these two possibilities, HeLa cells were infected for various times with either wild-type Ad 5 or one of the heterologous promoter mutants, then analyzed for expression of the E1B 58-kDa, E1B 19-kDa, and the E2 72-kDa, proteins as well as for expression of E1A. In an experiment comparing early gene expression in IEP548-1 and wild-type infections to that in β548-2 and IEP548-2 infections, we found the level of both the E2 and E1B proteins to be high regardless of the level of E1A expression (Fig. 6). In fact, quantitation of the autoradiographs shown in Fig. 6 revealed a comparable amount of E2 72-kDa present in cells infected with IEP548-1 and IEP548-2 despite a difference of at least 25-fold in E1A expression (Table 3). Although the levels of both E1B proteins in the reverse promoter mutant infections were nearly the same as those in wild-type infections, E1B proteins were significantly reduced in infections with the forward orientation HCMV IE promoter mutant. In a similar experiment comparing β548-1 to wild-type, β548-2, and IEP548-2, the results were essentially the same; however, the levels of E1B 58 and 19-kDa in β548-1 infections were not reduced to as great an extent as in IEP548-1 infections (Table 3). Although subsequent experiments have shown this reduction in E1B 58- and 19-kDa expression to be reproducible in cells infected with E1A-overproducing mutants, the reason for the reduction is not known at this time. It is possible that the high degree of transcriptional activity of the E1A gene somehow reduced the activity of the E1B transcription unit; however, in infections with IEP548-1 and β548-1 this low level of E1B expression apparently had no detrimental effect on replication. Nevertheless, it is clear from these data that mutants producing very low levels of E1A protein were capable of transactivating other early genes to the same, or nearly the same, extent as wild-type virus.

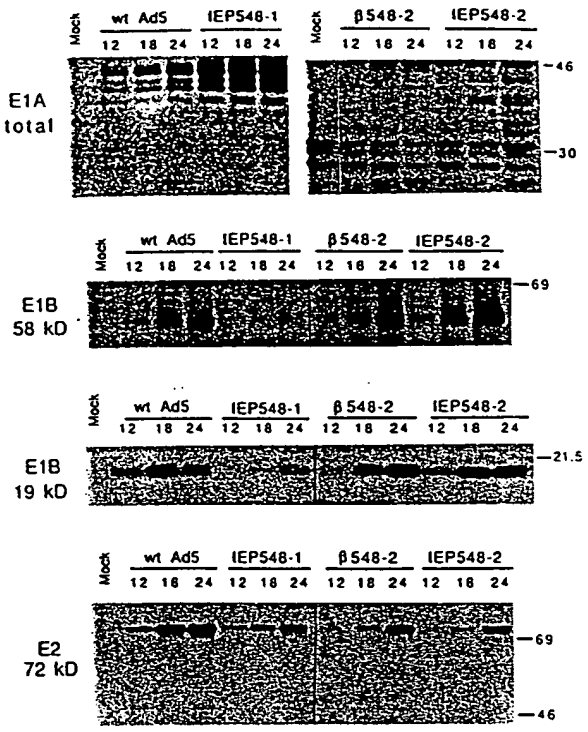

FIG. 6. Time course of early gene expression in infected HeLa cells. At 12, 18, and 24 hr postinfection, [^{35}S]methionine-labeled HeLa cell extracts were prepared as described under Materials and Methods. E1A, E1B 58-kDa, E1B 19-kDa, and E2 72-kDa proteins were immunoprecipitated with M73, 58C-2, 19C-24A, and H2-19 antibodies, respectively, then separated by SDS–PAGE. Molecular weights of markers are given in kDa at the right. The autoradiograph in the top right panel was exposed to the gel for five times longer than that shown in the top left panel.

DISCUSSION

We have constructed mutant adenoviruses by inserting strong heterologous promoters, derived either from the human β-actin gene or from the IE region of human cytomegalovirus, into a site upstream of the E1A coding sequence. The β-actin promoter has previously been shown in transient expression assays to direct expression of the bacterial chloramphenicol acetyltransferase gene at levels equal to or higher than does the SV40 early promoter in a wide variety of cell types (Gunning *et al.*, 1987; Sugiyami *et al.*, 1988). Consistent with these results, we have found this promoter capable of directing E1A production at levels three to five times higher than does the wild-type Ad 5 promoter, not only in HeLa cells as described here, but also in 293 and primary baby rat kidney (BRK) cells (unpublished results). In addition, we have shown that the HCMV immediate early promoter, reported to be several-fold stronger than the SV40 promoter (Boshart *et al.*, 1985), was considerably more active than the wild-type E1A promoter at driving E1A expression in

TABLE 3

EARLY GENE EXPRESSION IN HELA CELLS INFECTED WITH WILD-TYPE OR MUTANT VIRUSES

	Protein expression level[a]							
	E1A total		E1B-58 kDa		E1B-19 kDa		E2-72 kDa	
Virus Experiment:	1	2	1	2	1	2	1	2
Wild-type Ad 5								
12 hr p.i.	0.28	0.19	0.10	0.11	0.50	0.20	0.10	0.03
18 hr p.i.	0.45	0.37	0.56	0.45	1.3	1.1	0.71	0.51
24 hr p.i.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
β548-1								
12 hr p.i.	ND[b]	0.17	ND	0.06	ND	<0.05	ND	0.03
18 hr p.i.	ND	1.5	ND	0.17	ND	0.56	ND	0.25
24 hr p.i.	ND	1.8	ND	0.44	ND	0.85	ND	0.38
β548-2								
12 hr p.i.	<0.11	<0.10	0.04	0.11	0.05	0.15	0.01	0.01
18 hr p.i.	<0.11	<0.10	0.35	0.31	0.78	0.70	0.08	0.10
24 hr p.i.	<0.11	<0.10	1.1	0.74	1.9	1.4	0.42	0.35
IEP548-1								
12 hr p.i.	2.2	ND	0.01	ND	<0.05	ND	0.02	ND
18 hr p.i.	2.8	ND	0.02	ND	0.11	ND	0.11	ND
24 hr p.i.	2.8	ND	0.06	ND	0.32	ND	0.34	ND
IEP548-2								
12 hr p.i.	<0.11	<0.10	0.20	0.20	0.37	0.30	0.004	0.01
18 hr p.i.	<0.11	<0.10	0.53	0.85	1.4	1.1	0.04	0.19
24 hr p.i.	<0.11	<0.10	1.4	1.0	1.5	0.93	0.26	0.19

[a] The levels of gene expression were determined by densitometric scanning of the autoradiographs shown in Fig. 6 (Experiment 1) and a similar experiment in which early gene expression in β548-1 infected cells was also analyzed (Experiment 2). All values were normalized to the amount of the given protein expressed at 24 hr postinfection with wild-type Ad5 virus.

[b] Not determined.

HeLa cells, as well as 293 and BRK cells (latter data not shown). The full spectrum of E1A proteins displayed in wild-type Ad 5-infected cells was detected in cells infected with these mutant viruses, indicating that the increased level of E1A transcription driven by the heterologous promoters did not alter the normal patterns of alternative splicing of the E1A transcript or the post-translational modification of E1A proteins.

In our experiments, the increase in production of E1A proteins reflected an equivalent increase in expression of E1A at the transcriptional level. These results are quite different from those described by Solnick (1983) who found that when E1A was under control of the Ad 2 major late promoter (MLP), E1A transcription was enhanced 20- to 100-fold but E1A proteins were expressed at wild-type levels. The transcript encoding E1A, in that case, was actually a hybrid mRNA, the 5′ end of which was derived from the Ad 2 late leader sequence. The difference between Solnick's results and our own could possibly reflect a difference in translation efficiencies of transcripts containing β-actin, HCMV IE, or Ad 2 late leader sequences at the 5′ end. Alternatively, some translational or post-translational control of E1A production may be active late in infection, at which time the MLP-directed E1A product was expressed. The β-actin and HCMV IE promoters were shown to function early after infection, and thus would not have been subject to such regulation.

The overexpression of E1A gene products in β548-1 and IEP548-1 infections apparently did not lead to any alteration in the kinetics of replication in HeLa cells, indicating that E1A proteins may not be a limiting factor in wild-type virus replication. Evidence suggesting that E1A is produced in wild-type infections at a level exceeding that required for replication has also been reported by Osborne *et al.* (1982), who found that mutants lacking the E1A TATA box and the entire 5′ untranslated sequence expressed 5- to 10-fold reduced levels of E1A mRNA, but were fully capable of replicating in HeLa cells. Our mutants containing heterologous promoters in reverse orientation to the E1A transcription unit have allowed us to further extend those results. We were unable to detect E1A expression reproducibly either by primer extension of RNA or by immunoprecipitation of [^{35}S]methionine-labeled protein

from cells infected with these viruses. Nonetheless, growth curves for mutant virus replication in HeLa cells were indistinguishable from wild-type. Only in immunoprecipitations of [^{32}P]phosphate-labeled extracts from infection with one of the reverse promoter mutants (IEP548-2) was any E1A detected, albeit at levels 8-fold lower than in wild-type infections and 20-fold lower than in infections with the mutant containing the HCMV IE promoter in the same orientation as the E1A transcription unit. The level of E1A protein produced after infection with the reverse β-actin promoter mutant was reduced more than 14-fold with respect to wild-type and greater than 40-fold when compared to the forward β-actin promoter insertion mutant. It is clear from these results that viral replication in HeLa cells requires only a very low level of E1A expression, in fact, a level undetectable by most routine analyses. The capacity of reverse orientation promoter mutants to replicate does not appear to be limited to infection of HeLa cells, since the viruses are also replication proficient in BRK cells and the normal human diploid fibroblast line MRC5 (unpublished observations).

Our analysis of viral early gene expression in cells infected with reverse promoter mutants suggests that even greatly reduced levels of E1A protein are sufficient for wild-type levels of transactivation, which presumably leads to wild-type levels of viral replication. That replication with reverse orientation mutants was indeed dependent on transactivation by residual E1A protein was indicated by our results with a double mutant containing a termination in the 13 S mRNA. Our results on early gene expression are somewhat unexpected in light of a recent report by Brunet and Berk (1988) who found a direct relationship between E1A expression levels and early gene transcription. One possible reason for this discrepancy is that we have examined early gene expression at the protein level rather than at a transcriptional level, and the two may not give similar results. Alternatively, their study may have involved levels of E1A expression even lower than those we have obtained, thus within a linear range for transactivation of early gene transcription. Nonetheless, in our hands, low levels of E1A expression were sufficient both to transactivate adenovirus early genes and to induce viral replication at wild-type levels.

Our conclusions have been drawn by comparing overall levels of E1A proteins among the different recombinant viruses. These proteins are very heterogeneous and it is possible that only a small population of E1A proteins actually function in replication. The levels of functional E1A proteins may not differ substantially with the different mutant viruses, if, for example, a modification is required to generate functional E1A and the modification is rate limited by a cellular function, so that increasing the concentration of E1A proteins does not increase the amount of functional species. This possibility cannot be confirmed or excluded until the individual functions of the various E1A species have been unambiguously determined.

These observations provide a cautionary note to interpretation of results of studies with E1A mutants. Since the levels of wild-type E1A proteins can be reduced by as much as 10- to 20-fold without diminishing replication in HeLa cells, it is possible that E1A mutants scored as wild-type in their ability to replicate could have as little as 5–10% of wild-type E1A activity. By the same token, one is led to conclude that host range mutants isolated on the basis of their inability to replicate in HeLa cells must be severely reduced either in the levels of E1A expression or the activity of the mutant E1A proteins.

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